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RESEARCH ARTICLE

Ferulic Acid Released by Treatment with *Aspergillus oryzae* Contributes to the Cellular Antioxidant Capacity of Wheat Germ Extract

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Abstract An extract of fermented wheat germ (EFWG) was prepared by treatment with *Aspergillus oryzae* and *in vitro* antioxidant and cellular antioxidant capacities were measured. The induction of phase II detoxifying and antioxidant enzymes in exerting a cellular antioxidant capacity was determined. Treatment of wheat germ with *A. oryzae* significantly ($p<0.001$) increased the ferulic acid content, compared to controls, among 4 phenolic acids analyzed. The peroxyl radical-scavenging and reducing capacities and the cellular antioxidant capacity of EFWG were more potent than for extracts of wheat grain (EWG). The cellular antioxidant capacity of EFWG against AAPH and H_2O_2-induced oxidative stress was stronger than for EWG due to enhanced induction of the phase II enzymes HO-1, GST, and NQO-1. Potent *in vitro* and cellular antioxidant capacities of EFWG may result from an increased ferulic acid content due to treatment with *A. oryzae.*

Keywords: wheat germ, *Aspergillus oryzae*, ferulic acid, cellular antioxidant capacity

Introduction

Oxidative stress is an imbalance between generation of reactive oxygen species (ROS) and the capacities of the antioxidant defense system. Severe oxidative stress has been implicated in aging and chronic diseases, including cancer, diabetes, and coronary heart disease because of damage to lipids, proteins, carbohydrates, and DNA (1). ROS, such as the superoxide anion radical ($O_2^{\bullet -}$), hydroxyl radical ($OH^{\bullet}$), singlet oxygen (O_2^1), and hydrogen peroxide (H_2O_2) are generated as byproducts of normal cellular metabolism or as a result of exogenous factors including smoking and air pollution (2). Molecules that donate hydrogen or electrons to ROS can be used as scavengers to control oxidative stress-related diseases. Potential preventive capacities against oxidative stress have been reported from many kinds of natural compounds, including flavonoids and phenolic acids from medicinal herbs, fruits, and cereals (3,4).

Antioxidants can be divided into direct and indirect forms depending upon the mechanism of action (5). Direct antioxidants, which are always redox active, directly scavenge ROS and nitrogen radical species by being consumed or by chemical modification and must be replenished or regenerated. In contrast, indirect antioxidants may or may not be redox active, and impart antioxidant effects through stimulating the phase II detoxifying and antioxidant enzymes superoxide dismutase 1 (SOD-1), heme oxygenase-1 (HO-1), catalase (CAT), NAD(P)H: quinone oxidoreductase 1 (NQO-1), glutamylcysteine synthetase (GCS), and glutathione S-transferase (GST) (5). Some antioxidants, including phenolic Michael reaction acceptors (called bifunctional antioxidants) can exert antioxidant effects by both direct and indirect mechanisms (6).

Wheat (*Triticum* spp.) is a staple food worldwide. Wheat grain, used to make breads, cookies, cakes, pasta, and noodles, and other products, consists of an endosperm and bran, including a germ. Wheat germ, also known as wheat embryo, accounts for 2-4% of wheat grain (7). Most nutrients, except for starch, are concentrated in the germ (7). Wheat germ is especially rich in tocopherols and the

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vitamin B group (8). Even though wheat germ is a good source of vitamins and minerals, the germ is separated as a byproduct in the milling process and is mainly used as an animal feed because of adverse effects on the functional properties of dough. In addition, the relatively large amount of polyphenolic compounds in wheat germ is found in the form of insoluble bound hydroxycinnamic acids, mainly ferulic acid (9-11). Other non-nutrient components of wheat germ are methoxy-substituted benzoquinones as 2-methoxybenzoquinone and 2,6-dimethoxybenzoquinone, which have been reported to exert antiproliferative, antimetastatic, and immunological effects (12-15).

The purpose of this study was to investigate the effect of *Aspergillus oryzae* treatments on the *in vitro* and cellular antioxidant capacities of wheat germ. *A. oryzae* is known to produce extracellular enzymes, including feruloyl esterase, that release bound phenolic acids from the plant cell wall as free forms (16). Extracts of wheat germ (EWG) without treatment with *A. oryzae,* and extracts of fermented wheat germ (EFWG) after treatment with *A. oryzae* were prepared and analyzed for the phenolic acid content. The *in vitro* and cellular antioxidant capacities were determined, and a determination of whether phase II detoxifying antioxidant enzymes are induced was made using RT-PCR and western blot analysis.

Materials and Methods

Folin-Ciocalteu reagent, gallic acid, fluorescein, 2AAPH, Trolox, quercetin, neocuprine, MTT, 1,10-phenanthroline, Dulbecco's modified Eagle's medium (DMEM), Hank's balanced salt solution (HBSS), phosphate-buffered saline (PBS, pH 7.4), 2',7'-dichlorofluorescein-diacetate (DCFH-DA), dimethylsulfoxide (DMSO), and fetal bovine serum (FBS) were purchased from Sigma Co. (St. Louis, MO, USA). Malt extract broth (MEB) was purchased from MB Cell Co. (Los Angeles, CA, USA). *A. oryzae* (KCTC6377) was purchased from the Korea Collection for Type Culture (Biological Resource Center, Daejeon, Korea). Antibodies purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA) included HO-1 and NQO-1. The GSTα2 antibody was purchased from Cell Signaling Technology (Beverly, MA, USA). Human hepatoma (HepG2) cells were obtained from the American Type Culture Collection (ATCC, Rockville, MD, USA). Wheat germ was purchased from YoungNam Flour Mills Co. (Busan, Korea).

Preparation of EWG and EFWG Wheat germ (60 g) was soaked in 150 mL of distilled water for 4 h, followed by sterilization at 121°C for 30 min. *A. oryzae* cells were cultured with shaking in sterilized MEB medium at 30°C for 24 h. Fermented wheat germ was prepared via inoculation of sterilized wheat germ with *A. oryzae* cells (20:1) followed by culturing in an incubator (Vision Scientific Co., Ltd., Seoul, Korea) at 30°C for 72 h. EWG and EFWG were obtained by subjecting wheat germ and fermented wheat germ to extraction using 80% ethanol and lyophilization using a vacuum freeze dryer (Ilshinbiobase, Dongduchun, Korea). EWG and EFWG were kept at 20°C until analysis.

HPLC analysis of phenolic acids The phenolic acid contents of EWG and EFWG were analyzed using HPLC with a Zorbax Eclipse XDB-C18 column (4.6×50 mm; Agilent, Santa Clara, CA, USA). The mobile phase was composed of solvent A (0.02 M phosphate buffer, pH= 2.15) and solvent B (methanol: 0.02 M phosphate buffer, pH=2.15, 50:50, v/v) (17). The optimized gradient profile was linear from 0-10 min with 50 to 75% A at a flow rate of 0.5 mL/min; linear from 10-15 min with 75 to 100% A; 25 min of 100% A at a flow rate of 0.75 mL/min; then 30-50 min of 100% A at a flow rate of 1 mL/min. The injection volumes were 5 μL for standard solutions and sample extracts. A wavelength of 325 nm was used for detection of coumaric and ferulic acids. Syringic acid and vanillic acid were detected at 280 nm using a spectrophotometer.

Oxygen radical absorbance capacity assay An oxygen radical absorbance capacity (ORAC) assay was carried out using a Tecan GENios multi-functional plate reader (Salzburg, Austria) with fluorescent filters (an excitation wavelength of 485 nm and an emission filter of 535 nm) (18). In the final assay mixture, fluorescein (40 nM) was used as the target for the free-radical attack of AAPH (20 mM) as a peroxyl radical generator in a peroxyl radical-scavenging capacity assay (19). Trolox (1 μM) was used as a control standard and was prepared on a daily basis. The multi-functional plate reader (GENios; Tecan) was programmed to record fluorescence values of fluorescein every 2 min after addition of AAPH. All fluorescence measurements were expressed relative to the initial reading value. Final results were calculated on the basis of a difference in the area under the fluorescence decay curves between the blank and each sample. All data were expressed as micromole (μM) of Trolox equivalents (TE) where 1 ORAC unit was equivalent to the net protection area provided by 1 μM of Trolox.

Reducing capacity The electron donating capacities of samples to reduce Cu^{2+} to Cu^{+} were assessed according to the method of Aruoma *et al.* (20) An amount of 40 μL of different sample concentrations dissolved in ethanol were mixed with 160 μL of a mixture containing 0.5 mM $CuCl_2$ an 0.75 mM neocuproine, a Cu^{+} specific chelator, in a 10

mM phosphate buffer. Absorbance values were measured using a micro-plate reader (Tecan Co.) at 454 nm for 1 h. An increased absorbance value of the reaction mixture indicated a greater reducing power.

Cellular antioxidant capacity Cellular oxidative stress due to ROS generated using either AAPH or H_2O_2 was measured spectrofluorometrically following the DCFH-DA method (21). DCFH-DA diffuses through the cell membrane and is enzymatically hydrolyzed by intracellular esterase to non-fluorescent DCFH, which is rapidly oxidized to highly fluorescent DCF in the presence of ROS. HepG2 cells were first cultured in 96-well plates (5×10^5 /mL) with DMEM for 24 h. After the cells were incubated with different concentrations of sample dissolved in DMSO for 30 min, the medium was discarded, and the wells were gently twice washed using PBS. HBSS, which is fluorescently stable, was then added to each well instead of DMEM and AAPH and H_2O_2 were used as oxidative stress inducers. After cells were treated with either 60 μM AAPH or 1 mM H_2O_2 for 30 min, DCFH-DA was added to the culture plates at a final concentration of 40 mM and the cells were incubated for 30 min at 37°C in the dark. Quercetin (10 μM) was used as the positive control. After incubation, the cells were washed with HBSS, and the DCF fluorescence intensity was measured at an excitation wavelength of 485 nm and an emission wavelength of 535 nm using a Tecan GENios fluorometric plate reader.

Fluorescence microscopy analysis HepG2 cells were cultured in 12-well culture plates with cover slips. The cells were pretreated with 1-20 μM for 30 min. The cells were washed twice using HBSS and the medium was replaced with DMEM containing 50 μM AAPH for 30 min. After incubation, DMEM medium was replaced with HBSS containing 10 μM DCFH-DA, and the cells were kept for 30 min at 37°C in the dark, then fixed using 3.5% (v/v) formaldehyde for 30 min and visualized under a fluorescence microscope (Olympus Optical, Tokyo, Japan) at 200× magnification.

Transient transfection and an ARE-luciferase assay HepG2 cells were first cultured in 96-well plates (5×10^5 /mL) with DMEM for 24 h. Cells were then co-transfected for 24 h with 18 ng of the pGL 4.37 (*luc2P*/ARE/Hygro) vector as a control and 2 ng of the pGL 4.74 (*hRluc*/TK) vector as an internal control to determine the transfection efficiency in each well using FuGENE HD reagent (Promega, Madison, WI, USA). After HepG2 cells were incubated with different test sample concentrations dissolved in 10% DMSO for 24 h, the ARE-luciferase capacities were measured using a Dual-Glo luciferase assay system (Promega). In brief, multi-well plates containing HepG2 cells were removed from CO_2 incubator (Thermo Scientific, Waltham, MA, USA). For measurement of ARE luciferase capacity, a volume of Dual-Glo luciferase reagent (75 μL) equal to DMEM volume was added to each well, followed by gently mixing. After 10 min, a volume of Dual-Glo Stop & Glo reagent equal to the original culture medium volume was added to each well and gently mixed for measuring Renilla luciferase activity. Luminescence was measured using a Tecan GENios fluorometric plate reader. The ratio of the luminescence value from the experimental reporter to the luminescence value from the control reporter was calculated.

RT-PCR Total RNA was isolated using a Trizol reagent (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions. cDNA was generated from 1 μg of total RNA using the AccuPower CycleScript RT PreMix (Bioneer, Daejeon, Korea). PCR reactions were performed using the primers HO-1, 5'-GTGTAAGGACCC ATCGGAGA-3' (sense) and 5'-ATGACACCAAGGACC AGAGC-3' (antisense); for GCS, 5'-GGAAGGTGTGTTT CCTGGACT-3' (sense) and 5'-TATTATACCACGGGCTG AGAGG-3' (antisense); for NQO-1, 5'-CAGCGCCCCGG ACTGCACCAGAGCC-3' (sense); and 5'-GGGAAGCC TGGAAAGATACCCAGA-3' (antisense). The annealing temperatures was 60°C, except for HO-1. PCR products were analyzed using 1.5% agarose gel electrophoresis, stained with ethidium bromide (EtBr), and visualized using a UV detector (Vilber Lourmat, Torcy, France).

Western blot analysis HepG2 cells were lysed in radio immuno precipitation assay (RIPA) buffer (50 mM Tris-HCl) at pH 8.0, containing 1% NP-40, 0.5% sodium deoxycholate, 150 mM NaCl, and 1 mM PMSF and also a phosphatase inhibitor cocktail. Lysed cells were then subjected to SDS-PAGE and transferred to nitrocellulose membranes. The membranes were reacted with primary antibodies for 12 h, then incubated with appropriate horseradish peroxide-conjugated secondary antibodies for 1 h at room temperature. Proteins on the membranes were detected using a chemiluminescent detection kit (Intron Biotechnology, Seongnam, Korea) and visualized using the LAS4000 chemiluminescent image analyzer (Fuji, Tokyo, Japan).

Statistical analysis All data are presented as a mean± standard deviation (SD). Statistical analyses were carried out using the SPSS statistical package (Statistical Package for Social Science; SPSS Inc., Chicago, IL, USA) software and the significance of each group was verified based on a one-way analysis of variance (ANOVA) followed by either Duncan's test or Student's *t*-test. Statistical significance was defined as $p<0.05$ or $p<0.001$.

Table 1. Phenolic acid content of EWG and WFWG[1)]

Phenolic acid	EWG (mg/g)[2)]	EFWG (mg/g)
Coumaric acid	0.249±0.015	0.243±0.011
Ferulic acid	0.188±0.010	0.374±0.019***
Syringic acid	ND	ND
Vanillic acid	ND	ND

[1)]Results are expressed as the mean value of triplicate measurements with mean±SD.
[2)]EWG, extract of wheat germ; EFWG, extract of fermented wheat germ; ND, not detected; ***p<0.001 indicates a significant difference between EWG and EFWG based on Student's t-test.

Results and Discussion

Effect of an *A. oryzae* treatment on the phenolic acid content of EFWG The phenolic acid of wheat grain mainly consists of hydroxycinnamic acid (coumaric acid and ferulic acid) and hydroxybenzoic acid (syringic acid and vanillic acid) (17,22). The contents of the 4 phenolic acids (coumaric, ferulic, syringic, and vanillic acids) were measured using the HPLC method (17). Wheat germ contained only coumaric and ferulic acids (Table 1). After fermentation of wheat germ using *A. oryzae,* only the amount of ferulic acid was significantly (p<0.05) increased from 0.188 to 0.374% without any change in the amount of other phenolic acids, compared to controls. This increase in the soluble ferulic acid content was in agreement with another study that reported a microbial enzyme treatment generated ferulic acid from bound forms in wheat bran (23). As ferulic acid, the predominant hydroxycinnamic acid in wheat, is known to exist as a bound form, treatment with *A. oryzae* may be a potential method for releasing soluble ferulic acid from the bound forms in wheat germ.

Effect of an *A. oryzae* treatment on the *in vitro* antioxidant capacity of EFWG The antioxidant capacities of EWG and EFWG were investigated based on peroxyl radical-scavenging and reducing capacities. The scavenging capacities of EWG and EFWG in the presence of peroxyl radicals were dose-dependent at 1-50 mM (Fig. 1A). At a concentration of 1 mM, EWG showed 0.26 TE against peroxyl radicals generated using AAPH. In contrast, as a result of treatment with *A. oryzae*, the peroxyl radical-scavenging capacity of EFWG at 1 mM (0.71 TE) was relatively potent comparison to the capacity of EWG.

In order to confirm whether the antioxidant capacities of EWG and EFWG resulted from a capacity to donate electrons, reduction of Cu^{2+} to Cu^{+} was determined. With increasing concentrations of 1-50 mM, the reducing capacities of EWG and EFWG increased from 0 to 3.14 mM and from 0.17 to 6.79 mM at 1 and at 50 mM, respectively (Fig. 1B). At a concentration of 1 mM, EWG did not demonstrate any reducing capacity, while EFWG demonstrated

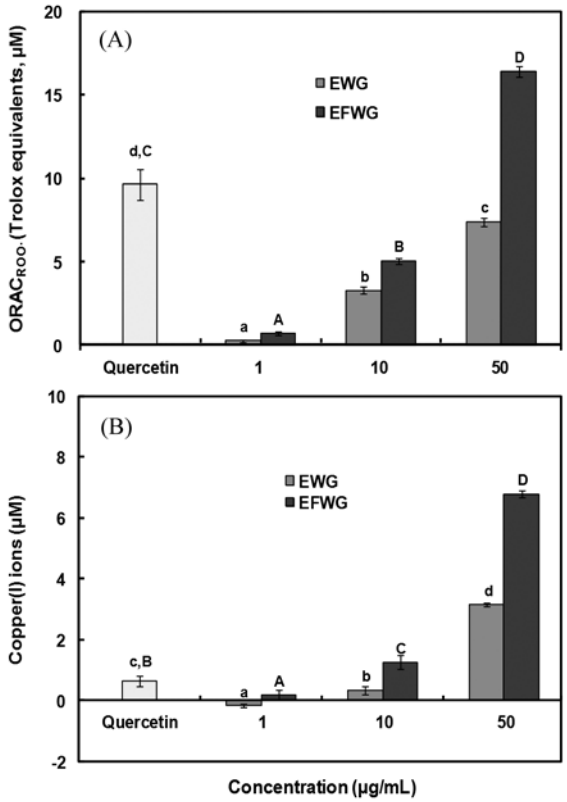

Fig. 1. Peroxyl radical-scavenging (A) and reducing capacities (B) of EWG and EFWG. Quercetin (1 mM) was used as a positive control. Data are expressed as a mean±SD of 3 individual experiments. Different corresponding letters indicate significant differences at p<0.05 based on Duncan's test.

a weak reducing capacity (0.17 mM), compared with Trolox (1.5 mM), a water-soluble α-tocopherol analogue used as a positive control. The increased amount in ferulic acid as a result of *A. oryzae* treatment may be responsible for the more potent ability of EFWG (compared with EWG) to donate hydrogens or electrons to peroxyl radicals for conversion to relatively stable compounds.

Effect of an *A. oryzae* treatment on the cellular antioxidant capacity of EFWG The cellular antioxidant capacities of EWG and EFWG were investigated using a cellular antioxidant capacity assay. HepG2 cells were pre-incubated with 1-200 mM EWG or EFWG for 30 min and exposed to 60 mM AAPH or 1 mM H_2O_2 for 30 min. Cells were then treated with DCFH-DA, which is a fluorescent probe that detects ROS, for 30 min to measure the level of intracellular oxidative stress that was induced. Fluorescence images of HepG2 cells treated with EWG or EFWG were displayed in Fig. 2A and 3A. The cellular oxidative stress levels in HepG2 cells were significantly (p<0.05) increased by 156.01 and 151.47% following treatments with AAPH and H_2O_2, respectively, compared with the control group (Fig. 2B, 3B). Quercetin (10 mM), used as a positive control,

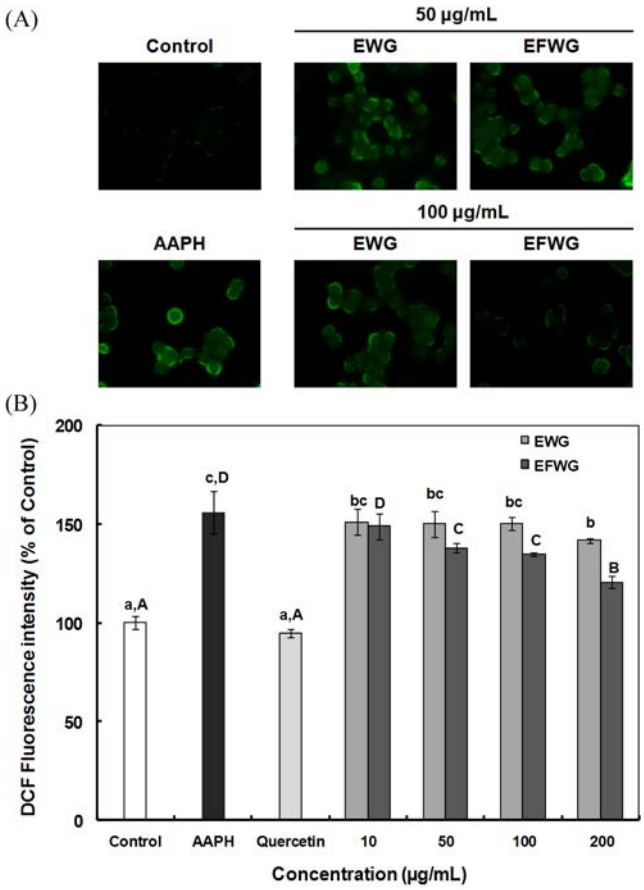

Fig. 2. Cellular antioxidant capacities of EWG and EFWG in AAPH-induced HepG2 cells based on fluorescent microscopic analysis (A) and a DCFH-DA assay (B). Fluorescent microscopic analysis for ROS observation was performed using DCFH-DA stained HepG2 cells after treatment with 50 or 100 mg/mL of EWG and EFWG in the absence and presence of 60 mM AAPH. Quercetin (10 mM) was used as a positive control. Data are expressed as percentages of the value of untreated cells (mean± SD, n=3). Different corresponding letters indicate significant differences at p<0.05 based on Duncan's test.

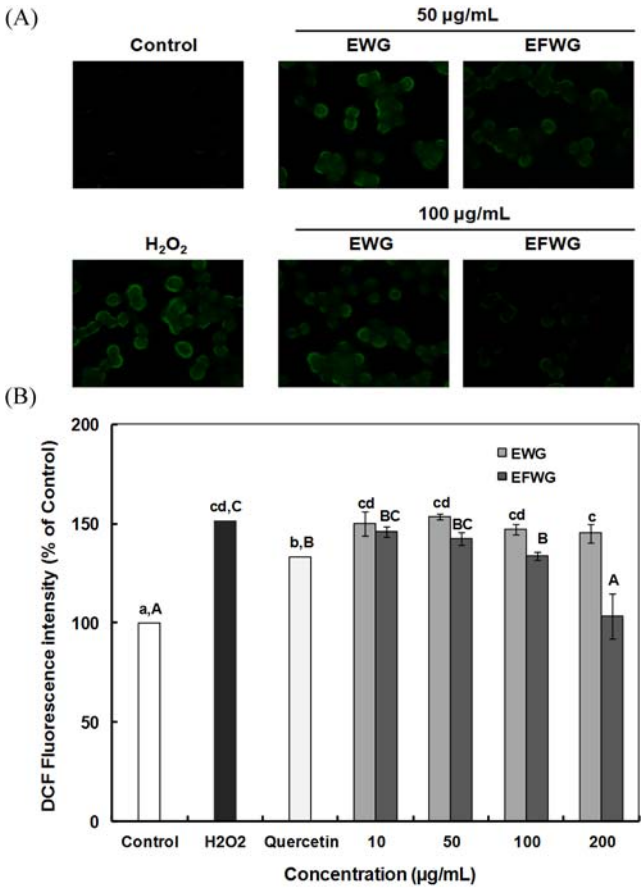

Fig. 3. Cellular antioxidant capacities of EWG and EFWG in H_2O_2-induced HepG2 cells based on fluorescent microscopic analysis (A) and a DCFH-DA assay (B). Fluorescent microscopic analysis for ROS observation was performed using DCFH-DA stained HepG2 cells after treatment with 50 or 100 mg/mL of EWG and EFWG in the absence and presence of 1 mM H_2O_2. Quercetin (10 mM) was used as a positive control. Data are expressed as percentages of the value of untreated cells (mean± SD, n=3). Different corresponding letters indicate significant differences at p<0.05 based on Duncan's test.

significantly (p<0.05) decreased the level of intracellular oxidative stress caused by AAPH and H_2O_2 to 94.52 and 133.33%, respectively, compared with controls. The intracellular oxidative stress induced by AAPH and H_2O_2 was dose-dependently alleviated by EFWG at 10-200 mM, whereas the stress level was reduced by EWG only at 200 mM. This dose-dependency of the cellular antioxidant capacity of EFWG between 50 and 100 mM also appeared under fluorescence microscopy observation (Fig. 2A, 3A). In comparison with EWG, the cellular antioxidant capacity of EFWG against the oxidative stress induced by AAPH and H_2O_2 observed at 50-200 mM was more potent. In addition, the cellular antioxidant capacities of EWG and EFWG were very weak when compared to quercetin. The significant (p<0.05) difference between the EWG and EFWG cellular antioxidant capacities may be ascribed to soluble ferulic acid that is released by the *A. oryzae* treatment that penetrates the cell membrane to suppress the intracellular oxidative stress that is induced by peroxyl radicals and H_2O_2.

The cellular antioxidant capacity of antioxidants consists of either direct or indirect antioxidant mechanisms (5,24). For instance, the cellular antioxidant capacity of sulforaphane may be due to an indirect antioxidant capacity that induces phase II antioxidant and detoxifying enzymes because the *in vitro* antioxidant capacity of sulforaphane is weak (5). However, EFWG showed intermediate peroxyl radical-scavenging and reducing capacities, indicating that the cellular antioxidant capacity of EFWG probably results from the combined effect of both direct and indirect antioxidant capacities.

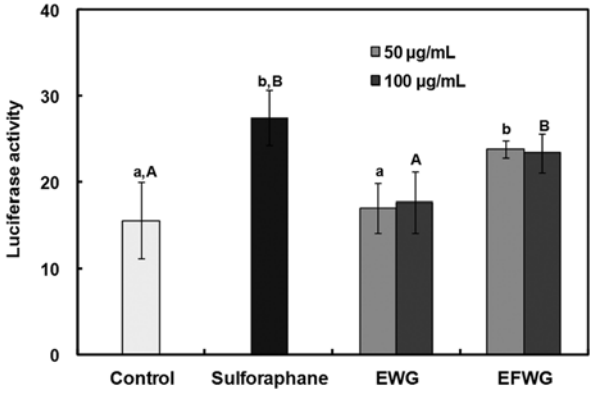

Fig. 4. Effect of EWG and EFWG on activation of ARE related genes in HepG2 cells. Sulforaphane (1 mM) was used as a positive control. Data are expressed as a mean±SD of 3 individual experiments. Different corresponding letters indicate significant differences at $p<0.05$ based on Duncan's test.

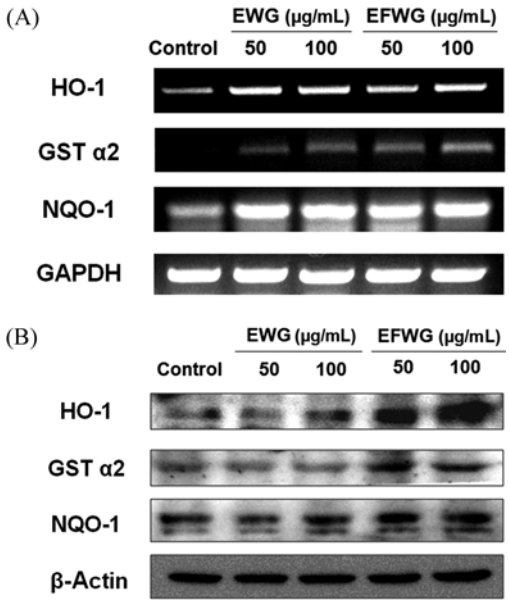

Fig. 5. Effect of EWG and EFWG on transcriptional (A) and translational (B) capacities of detoxifying and antioxidant enzymes in HepG2 cells.

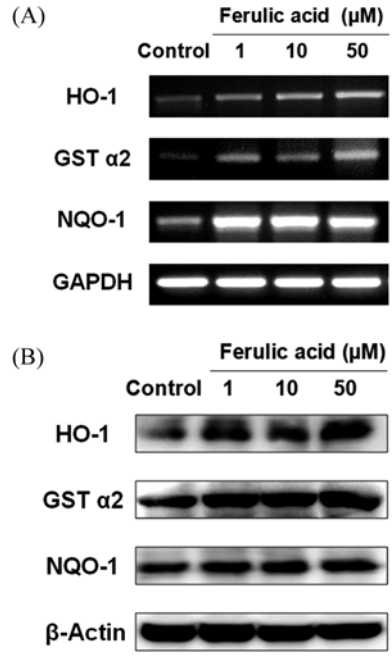

Fig. 6. Effect of ferulic acid on transcriptional (A) and translational (B) capacities of detoxifying and antioxidant enzymes in HepG2 cells.

Contribution of the ferulic acid released by *A. oryzae* to the cellular antioxidant capacity via induction of phase II detoxifying and antioxidant enzymes To check whether EWG and EFWG, as indirect antioxidants, stimulate transcription of ARE-related genes in HepG2 cells, a luciferase reporter plasmid carrying the ARE promoter was introduced to HepG2 cells. ARE is a *cis*-acting DNA regulatory element present in the promoter/enhancer regions of genes that encodes many antioxidant and detoxifying enzymes. A luciferase assay showed that only EFWG (50-100 μM) significantly ($p<0.05$) increased the ARE promoter capacity, compared with the control (Fig. 4). An enhanced ARE promoter capacity of sulforaphane used as a positive control for indirect antioxidant capacity testing was also observed. The transcriptional level of phase II antioxidant and detoxifying enzymes after treatment with EFWG is shown in Fig. 5A. The antioxidant enzyme HO-1 and the detoxifying enzymes NQO-1 and GST were transcriptionally stimulated. An increase in the translational level of phase II antioxidant and detoxifying enzymes was observed in HO-1, GST, and NQO-1 (Fig. 5B).

To verify involvement of ferulic acid in the cellular antioxidant capacity of EFWG, the transcriptional and translational levels of HO-1, GST, and NQO-1 were evaluated. Dose-dependent stimulation of HO-1, GST, and NQO-1 transcription by ferulic acid was observed (Fig. 6A). Induction of the HO-1 gene by ferulic acid has also been confirmed in lymphocytes and umbilical vein endothelial cells (25,26). In addition, in this study ferulic acid induced translation of HO-1, GST, and NQO-1 in a dose-dependent manner (Fig. 6B), which is consistent with another study showing an increase in the translational level of HO-1 in human umbilical vein endothelial cells (26).

EFWG can apparently exert a cellular antioxidant capacity via up-regulation of transcription and translation of the phase II antioxidant and detoxifying enzymes HO-1, GST, and NQO-1. This induction effect on the antioxidant-related gene by ferulic acid was confirmed in another study using murine hepatoma cells where the ferulic acid isolated from green onions was active in inducing NQO-1 (27). The inducing effect of ferulic acid on phase II antioxidant and detoxifying enzymes has also been identified in animal

studies (28).

In conclusion, EFWG prepared via treatment with *A. oryzae* had a higher phenolic acid content, especially ferulic acid, and higher *in vitro* antioxidant activity such as peroxyl radical-scavenging and reducing capacities than EWG. Also, the cellular antioxidant capacity of EFWG, examined using stimulation of the phase II detoxifying and antioxidant enzymes HO-1, GST, and NQO-1 against AAPH- and H_2O_2-induced oxidative stress, was more potent than for EWG. The enhanced *in vitro* and cellular antioxidant capacity of EFWG may be due to an increase in the ferulic acid content caused by treatment with *A. oryzae.* These *in vitro* and cellular antioxidant capacities of EFWG can provide a scientific basis for further animal and clinical studies.

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Disclosure The authors declare no conflict of interest.

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