Laccase Fermentation of Clove Extract Increases Content of Dehydrodieugenol, Which Has Neuroprotective Activity against Glutamate Toxicity in HT22 Cells

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Introduction

Food processing techniques such as fermentation, extrusion, and roasting have been used to improve the nutritional values, flavors, and storage period of foods [1]. Recently, it has been demonstrated that simple food processing techniques, especially fermentation, could induce changes in the content of biologically active compounds in certain oriental herbal medicines. For example, the crude enzyme extract derived from *Aspergillus kawachii* significantly increased the content of neuroprotectants such as liquiritigenin and quercetin, in the extracts of licorice root and onion, respectively [1−3]. In addition, fermentation of *Daldinia eschscholzii* by laccase induced the production of immunosuppressive compounds such as dalesconols A and B [4]. Therefore, the development of simple fermentation techniques may be helpful in improving the biological activities of food and herbal medicines.

Laccase (a *p*-diphenol:dioxygen oxidoreductase), an enzyme belonging to the family of multicopper oxidases, catalyzes the one-electron oxidation of various substrates coupled with the reduction of oxygen to water [5]. It was first identified in the exudates of the Japanese lacquer tree (*Rhus vernicifera*) and has been found in various fungi [5], including *Trametes versicolor* [6], which is commonly known as the polypore mushroom or turkey tail. Recently, there have been many studies investigating its general [7, 8], catalytic [9−11], and biological [12, 13] properties. In particular, it was demonstrated that laccase oxidation generates the reactive phenoxyl radicals that can lead to oxidative polymerization, resulting in the formation of oligomeric compounds [14, 15]. A wide range of molecules, including di- and polyhydrated monoaromatic, methoxyphenolic, and monohydroxylated biarylic compounds, such as catechol, hydroquinone, vanillic acid, syringic acid, hydroxylated biphenyls, and ferulic acid, have been identified as laccase substrates [16].

Although glutamate is known as an excitatory neurotransmitter in mammals, it can also induce oxidative stress that leads to neurodegeneration when present in...
high concentrations [17]. It has been reported that glutamate toxicity leads to the depletion of glutathione in cells, resulting in the inhibition of cystine uptake through the glutamate/ cystine antiporter [17]. This phenomenon promotes the cell death signaling cascades, involving the Ca\(^{2+}\) influx, intracellular reactive oxygen species (ROS) generation, and lipid peroxidation [18, 19]. Thus, we hypothesized that fermentation of plant extracts may induce an increase in the content of antioxidative constituents that can protect neuronal cells against glutamate-mediated damage.

As a preliminary screening, extracts of 10 different plants (i.e., Aralia cordata (root), Syzygium aromaticum (bud), Citrus unshiu (peel), Glycyrrhiza uralensis (root), Melia azedarach (fruit), Piper nigrum (fruit), Rehmannia glutinosa (root), Rubia akane (leaf), Sálvia miltiorrhiza (root), and Schizandra chinensis (fruit)) were prepared for experimentation. These extracts were subjected to fermentation with laccase derived from Trametes versicolor (LTV) to induce changes in their molecular constituents and to improve their neuroprotective effects. To our best knowledge, this study is the first to report that the fermented Syzygii Flos (clove) extract showed a significant change in high-performance liquid chromatography (HPLC) profile and enhanced protection of mouse hippocampal neuronal cells (HT22) against glutamate-induced cell death. In addition, we optimized the LTV-catalyzed clove fermentation condition, isolated and quantified the modified constituents, and compared the HT22 cell protective effects of the isolated compounds.

Materials and Methods

General
Organic solvents for extraction and chromatography, including n-hexane, ethyl acetate (EtOAc), ethanol (EtOH), and methanol (MeOH), were purchased from Duskan Pure Chemicals (Korea). HPLC-grade acetonitrile (MeCN) and acetic acid (HOAc) were obtained from Merck (Germany). Nuclear magnetic resonance (NMR) analysis for compound identification was performed on a Bruker Avance400 Digital NMR spectrometer (Bruker, Germany), and the chemical shifts (δ) were expressed in parts per million (ppm) relative to the tetramethylsilane internal standard. To monitor the elution pattern, thin-layer chromatography was performed using RP-18 F254S and silica gel 60 F254 plates (Merck, Germany) for reverse and normal phase chromatography, respectively. 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT; Amresco, USA) was used to estimate cell viability. 5-(and-6)-Chloromethyl-2′,7′-dichlorodihydrofluorescein diacetate, acetyl ester (CM-H\(_2\)DCFDA), 4,4-difluoro-5-(4-phenyl-1,3-buta dienyl)-4-bora-3a,4a-diaza-s-indacene-3-undecanoic acid (BODIPY581/ 591 C\(_{11}\)), and Fluo-4 AM were purchased from Invitrogen (USA) for flow cytometry analysis.

Plant Materials and Extraction
Plants were purchased from a local market in Daegu, Korea, and voucher specimens were deposited at the Laboratory of Natural Products Medicine, College of Pharmacy, Kyungpook National University. The plants were refluxed with 95% EtOH for 3 h, and the extracted solutions were filtered and evaporated for fermentation and examination of their neuroprotective effects.

Fermentation of Plant Extracts with Laccase
Laccase (20 mg, 13.6 U/mg) from T. versicolor (LTV; Sigma, USA) was suspended in 50 ml of acetic acid solution (pH 4.5) before mixing with each ethanolic plant extract (50 mg). The mixtures were incubated at 25°C for 1 h, and the reacted materials were partitioned with 50 ml of EtOAc. The EtOAC soluble fractions were concentrated for HPLC analysis and the examination of their neuroprotective effects.

HPLC Analysis of Plant Extracts
The EtOAc extracts were dissolved in MeOH to prepare 5 mg/ml solutions. The solutions were filtered through a 0.45 μm membrane filter (Millipore, USA) and the filtrates were analyzed by HPLC (Jasco, Japan) to compare the chromatographic pattern with non LTV-treated extracts (controls). The HPLC system was equipped with a PU-2080 Plus Intelligent HPLC pump and a MD-2010 Plus Multiwavelength detector with the wavelength set at 254 nm. An AXTerra RP18 column (4.6 x 250 mm, 5 μm; Waters, USA) was used as the stationary phase, and water (solvent A) and MeCN (solvent B), each containing 1% HOAc, were used as the mobile phases. After sample injection into the column, the concentration of solvent B was increased from 5% to 100% in 50 min, held at 100% for 5 min, decreased to 5% in 5 min, and held at 5% for 10 min for equilibration. The flow rate was 0.8 ml/min.

Optimization of Clove Fermentation Time
To find the optimal fermentation time of clove extract, changes in the major peaks during fermentation were monitored over time. Clove ethanolic extract (2 g) was suspended in 20 ml of distilled water and 1 ml aliquots of suspension were each added to 100 ml aliquots of LTV solution (5.44 U/ml) in acetic acid solution (pH 4.5). The mixtures were incubated at 25°C for 0.5, 1, 2, 4, 6, and 17 h. The inactivated LTV-treated extract was considered as zero time. The incubated samples were extracted with 100 ml EtOAc and concentrated. The residues were dissolved in MeOH (5 mg/ml) and 10 μl samples were used for HPLC analysis, in which the concentration of solvent B was increased from 30% to 80% in 30 min, held at 80% for 5 min, decreased to 30% in 5 min, and held at 30% for 10 min.

Bulk Fermentation of Clove Extract
In order to isolate the compounds that decreased or increased in content in clove extract after fermentation with LTV, bulk
fermentation of the extract was performed. The ethanolic extract of clove (30.8 g) was suspended in 500 ml of acetic acid solution (pH 4.5) and treated with 500 ml of LTV (1 g, 13.6 U/mg) solution at 25°C for 1 h. Then, the EtOAc soluble fraction was prepared.

Isolation and Identification of Compounds
The EtOAc soluble fraction (10.7 g) of the bulk fermented clove was applied to a silica gel column (Ø8 × 21 cm) and eluted with mixtures of n-hexane and EtOAc (20:1 to 3:1) to yield seven fractions (Fr. 1–7). Fr. 2 and Fr. 6 gave compounds 1 (1.2 g) and 2 (186.0 mg), respectively, which were identified as eugenol and dehydrodieugenol, respectively, by comparing our data with those in the literature [20, 21].

Eugenol (1). $^1$H NMR (500 MHz, CDCl$_3$): δ 6.68 (1H, d, $J$ = 8.3 Hz, H-5), 6.68 (1H, dd, $J$ = 8.3 and 1.8 Hz, H-6), 6.67 (1H, d, $J$ = 1.8 Hz, H-2), 5.94 (1H, m, H-8), 5.49 (1H, brs, -OH), 5.06 (2H, m, H-9), 3.95 (3H, s, -OCH$_3$), and 3.79 (2H, dt, $J$ = 6.8 and 2.0 Hz, H-7). $^{13}$C NMR (125 MHz, CDCl$_3$): δ 146.40 (C-3), 137.89 (C-8), 131.89 (C-1), 121.14 (C-6), 115.49 (C-5), 114.21 (C-2), 111.07 (C-9), 55.82 (-OCH$_3$), and 39.62 (C-7).

Dehydrodieugenol (2). $^1$H NMR (500 MHz, CDCl$_3$): δ 6.73 (2H, d, $J$ = 2.0 Hz, H-6 and 6'), 6.71 (2H, d, $J$ = 2.0 Hz, H-2 and 2'), 6.00 (2H, brs, -OH), 5.91–6.01 (2H, m, H-8 and 8'), 5.03–5.12 (4H, m, H-9 and 9'), 3.89 (6H, s, -OCH$_3$), and 3.55 (4H, d, $J$ = 6.8 Hz, H-7 and 7'). $^{13}$C NMR (125 MHz, CDCl$_3$): δ 147.2 (C-3 and 3'), 140.8 (C-4 and 4'), 137.6 (C-8 and 8'), 131.8 (C-1 and 1'), 124.3 (C-5 and 5'), 123.0 (C-6 and 6'), 115.6 (C-9 and 9'), 110.6 (C-2 and 2'), 56.0 (2× -OCH$_3$), and 39.9 (C-7 and 7').

Calibration Curves for Isolated Compounds
To quantify the isolated compounds 1 and 2, HPLC analysis was performed as described above. To prepare stock solutions, 1 mg of each compound was dissolved in 1 ml of MeOH, and serially diluted with MeOH. Ten microliters of each standard solution was injected into the column. Calibration curves were drawn by plotting the peak area (Y) against the amount of each sample in micrometers (X). The calibration curves for 1 and 2 were $Y = 2.1553X + 0.6528$ ($r^2 = 0.9998$) and $Y = 15.4740X + 0.3850$ ($r^2 = 0.9998$), respectively.

Cell Culture and MTT Cell Viability Assay
The mouse-derived hippocampal neuronal cell line (HT22) was donated by Prof. Dong-Seok Lee (College of Natural Sciences, Kyungpook National University) and cultured in Dulbecco’s modified eagle’s medium (DMEM; Welgene, Korea) supplemented with 10% fetal bovine serum (Gibco, USA), 100 U/ml penicillin, and 100 μg/ml streptomycin (Welgene) under 5% CO$_2$ with humidified air at 37°C.

The cell viability assay was performed as described previously [22]. Briefly, the cells were seeded at a density of 3 × 10$^4$ cells/ml in a 24-well plate (BD Biosciences, USA). After 12 h of incubation, the cells were treated with test samples for 12 h before induction of cytotoxicity using 5 mM glutamate (12 h). The medium was removed and 500 μl of MTT (0.5 mg/ml in phenol red-free DMEM) was added for 4 h. The purple formazan crystals produced by live cells were dissolved in 500 μl of dimethyl sulfoxide, and the optical density (OD) was measured at 575 nm after shaking the culture plates for over 1 h at room temperature. Cell viability was expressed as the percentage of OD relative to the OD of the control (CNT, 100%). The antioxidant quercetin (10 μM), which was reported as an HT22 cell protectant against 5 mM glutamate [3], was used as a positive control.

LDH Leakage Assay
Examination of cell damage through the cytosolic lactate dehydrogenase (LDH) leakage assay was performed using a CytoTox96 non-radioactive cytotoxicity assay kit (Promega, USA), according to the manufacturer’s protocol.

Flow Cytometry Analysis
Flow cytometry analysis to measure the Ca$^{2+}$ influx, intracellular ROS generation, and lipid peroxidation, using Fluo-4 AM, CM-H$_2$DCFDA, and BODIPY581/591 C$_2$, respectively, was performed on 10,000 viable cells with a FACS ARIA III system (BD Biosciences) as previously described [23].

Fermentation of Clove Extract with Various Laccases
Clove ethanolic extract (2 g) was suspended in 40 ml of distilled water and 2 ml suspensions were treated with 544 U of various laccases derived from T. versicolor (LTV), Pleurotus ostreatus (LPO), and Rhus vernicifera (LTV). LTV and LPO were suspended in 100 ml of acetic acid solution (pH 4.5) for fermentation, whereas LRV was suspended in 100 ml of phosphate buffer (20 mM, pH 6.5). The mixtures were incubated at 25°C for 1 h, before the EtOAc-soluble fractions were collected and analyzed by HPLC (5 mg/ml, 10 μl injections).

Statistical Analysis
All experiments were performed at least in triplicate, and the data were expressed as the mean ± standard deviation (SD). Statistical significance was determined by one-way analysis of variance. A value of $p < 0.05$ was considered to be significant.

Results
Effect of Fermentation of Various Plant Extracts with LTV Changes in the HPLC profiles. The HPLC profiles of LTV-treated plant extracts were compared with those of non-LTV-treated ones. The HPLC chromatograms of Syzygium aromaticum (clove), Glycyrrhiza uralensis, Piper nigrum, and Rehmannia glutinosa were significantly changed after fermentation, whereas those of Aralia cordata, Citrus unshiu, Melia azedarach, and Rubia akane were slightly altered. On the other hand, the HPLC profiles of Salvia miltiorrhiza and Schizandra chinesis were unaffected (data not shown).
Changes in the Neuroprotective Activity. The eight plant extracts that showed changes in their HPLC profiles after LTV fermentation were used to treat HT22 cells to examine their neuroprotective effects. As shown in Fig. 1A, the viability of cells treated with LTV-fermented clove extract (10 μg/l) was significantly increased to 99.04 ± 3.42%, whereas that of the 5 mM glutamate-treated group was 28.03 ± 2.34% (CNT; 100.00 ± 3.00%). This neuroprotective action of LTV-fermented clove extract was 62.35% greater than its unfermented counterpart.

Neuroprotective Effect of LTV-Treated Clove Extract

After the preliminary screening, inactivated LTV- and LTV-treated clove extracts were added to HT22 cells in various concentrations, ranging from 1 to 25 μg/l. When the cells were exposed to 5 mM glutamate, the cell viability was reduced to 17.86 ± 0.45% compared with the CNT (100.00 ± 3.00%). In contrast, the LTV-treated clove extract increased the cell viability in a concentration-dependent manner. In particular, the viability of HT22 cells treated with LTV-fermented clove was 99.54 ± 2.11% at 10 μg/l, whereas the viability of cells exposed to inactivated LTV-treated clove extract was 21.64 ± 2.07% (Fig. 1B).

Optimization of Fermentation Time

Upon treatment of clove extract with LTV for 0.5, 1, 2, 4, 6, and 17 h, the major peak at zero time (A, Rt 16.3 min) was reduced with increasing fermentation time. In contrast, peaks B (Rt 27.1 min), C (Rt 28.4 min), and D (Rt 36.5 min) were newly detected after fermentation. The clove extract fermented with LTV for 1 h showed the best production of peak B. However, the signal of the peak was decreased time-dependently after 1 h (Figs. 2A and 2B). This is presumably due to polymerization by the continuous radical reaction by LTV. Therefore, the optimal fermentation time for clove extract was determined as 1 h.

Verification of Isolated Compounds in the Fermented Clove Extract

To identify the compounds that had increased or decreased in content after fermentation, column chromatography and NMR analysis were performed. The chemical structures of 1 and 2 are shown in Fig. 2C. The presence of 1 and 2 was verified by comparing the retention times of their peaks in
fermented clove extract with those of the isolated standards. The increased peak B was identified as dehydrodieugenol (2, DE) by NMR analysis, whereas the decreased peak A was confirmed as eugenol (1, EU) (Fig. 2C). Quantification of these compounds was performed using calibration curves (Fig. 2D), where DE peaked at 1 h after fermentation to 103.50 ± 8.20 mg/g ex (DE was not detected at zero time), while EU was decreased to 79.54 ± 4.77 mg/g ex (185.41 ± 10.16 mg/g ex at zero time). The quantification of peaks C–E could not be performed as they were present in trace amounts. The trace peaks produced by the laccase treatment are assumed to be one of the following eugenol dimers such as 2A and 2B, considering the mechanism of oxidative coupling reaction of eugenol (Fig. 3). These minor compounds will be identified by comparing their LC-MS profiles with those of synthetic ones in future works.

Effects of EU and DE on Glutamate-Induced Cell Death

In order to examine the neuroprotective effects of EU and DE, HT22 cells were pretreated with these compounds before induction of cell death using 5 mM glutamate. When HT22 cells were stressed with 5 mM glutamate for 12 h, the cells exhibited shrunken morphology. In contrast, cells treated with 10 μM DE were healthy, as were the cells in the 10 μM quercetin-treated group (Fig. 4A). In the MTT cell viability assay, treatment of DE led to recovery in cell viability in a concentration-dependent manner; 10 μM DE significantly increased cell viability to 84.60 ± 2.40% (CNT, 100.00 ± 1.99%; 5 mM glutamate only, 24.66 ± 1.05%). In contrast, EU showed negligible neuroprotective activity at
the same concentration (24.74 ± 2.27%; Fig. 4B). Glutamate increased cytosolic LDH leakage into the extracellular matrix by 10.28 ± 0.69-fold over the CNT (1.00 ± 0.35-fold), whereas 10 μM DE significantly attenuated the LDH leakage to 0.43 ± 0.17-fold. On the other hand, EU did not show any significant inhibitory effect on LDH leakage (Fig. 4B). Based on these results, the two compounds were tested at the same concentrations of 10 μM in the subsequent experiments.

**Effects of EU and DE on Ca^{2+} Influx, Intracellular ROS Generation, and Lipid Peroxidation**

As shown in Fig. 4C, Ca^{2+} influx in HT22 cells was significantly elevated by 2.00 ± 0.10-fold upon glutamate exposure compared with that in CNT (1.00 ± 0.03-fold) cells. In contrast, DE effectively suppressed the glutamate-induced Ca^{2+} influx to 0.91 ± 0.02-fold at 10 μM. Pretreatment of cells with 10 μM DE also remarkably attenuated the increase in intracellular ROS generation induced by glutamate toxicity (2.18 ± 0.07-fold compared with CNT) to 1.57 ± 0.12-fold. Moreover, the glutamate-induced lipid peroxidation (11.50 ± 3.76-fold) in the cells was similarly significantly reduced by 10 μM DE treatment to 2.02 ± 0.07-fold. On the contrary, the effect of EU against glutamate toxicity in HT22 cells was negligible.

**Comparison of Various Laccase Treatments of Clove Extract**

The LTV- and LPO-treated clove extracts showed significant changes in their HPLC profiles (Fig. 5A). Moreover, improved HT22 cell viability was also observed after fermentation with LTV (74.04 ± 3.58%) and LPO (42.01 ± 2.75%) at a concentration of 10 μg/l, respectively (CNT, 100.00 ± 4.38%; 5 mM glutamate, 28.75 ± 1.32%). However, the neuroprotective effect of LRV-treated clove extract was negligible (Fig. 5B). Among the three laccases, LTV induced the most drastic change in the HPLC pattern and neuroprotective activity of clove extract.

**Discussion**

Syzygii Flos (clove) has been used for fragrance and in cooking as a spice [24], and exhibits a variety of biological activities, such as antioxidative and anti-inflammatory effects [25, 26]. Clove produces an essential oil containing eugenol, eugenol acetate [27], acetyleneugenol, isoeugenol, and methyleugenol [28]; these molecules have the phenyl propanoid backbone. Theoretically, these compounds could act as substrates for laccase in a polymerization reaction owing to the presence of methoxyl- and monohydroxylated phenolic groups in their structures.

In this study, it was demonstrated that the composition of constituents in clove extracts was significantly altered after fermentation with laccase derived from *T. versicolor*. The content of the monomer form eugenol (1, EU) was reduced in the fermented clove extract, whereas that of the dimer dehydrodieugenol (2, DE) was increased. This result indicated that 1 was a suitable substrate for LTV and its reaction led to the production of 2, in accordance with previous findings [14, 15].

In a previous report, 2 was isolated from an ethanolic clove extract and its hypoglycemic effects in genetically diabetic KK-A' mice was investigated [29]. Furthermore, it also exhibited a promising antidepressant-like effect in
mice forced to perform a swimming test [30]. However, the protective effect of 2 on HT22 cells against high concentrations of glutamate has not been reported, to the best of our knowledge.

Hosny and Rosazza [31] investigated the oxidation of (+)-catechin by laccase and horseradish peroxidase and discovered that the products markedly inhibited iron-induced lipid peroxidation in rat brain homogenates as well as Fe$^{2+}$-adenosine 5′-diphosphate sodium salt (ADP)/nicotinamide adenine dinucleotide phosphate reduced (β-NADPH) in rat liver microsomes. These oxidation products had lower half maximal inhibitory concentration $(IC_{50})$ values for thiobarbituric acid reactive substance formation than catechin [31]. In our case, it was proposed that the increased number of phenolic hydroxyl groups in the dimer form 2 contributed to the stronger protective effect since phenolic groups generally act as ROS scavengers [32].

The results presented in this study suggested that the neuroprotective action of clove extract was significantly
improved by simple laccase fermentation that led to an increase in 2 content. These findings can be helpful in the development of functional foods for the treatment of various neurodegenerative diseases.

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Conflict of Interest

The authors have no financial conflicts of interest to declare.

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