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Isolation of caffeic acid from *Perilla frutescens* and its role in enhancing γ-glutamylcysteine synthetase activity and glutathione level

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ABSTRACT

Perilla frutescens is an annual herbaceous plant native to Asia, where its leaves are used in Asian gourmet food. Our previous study showed that the inhibition of γ-glutamylcysteine synthetase (γ-GCS) activity was remarkably recovered by pretreatment with perilla leaf extract (PLE). The objective was to fractionise PLE, and to identify the active component that is responsible for the enhancement of γ-GCS activity and glutathione (GSH) concentration. Among the five fractions from PLE, PLE-III of the ethyl acetate fraction showed the highest γ-GCS activity in a HepG2 cell experiment, and was further chromatographed. The purified compound, which enhanced γ-GCS activity, was finally identified as caffeic acid. We first report the enhancement of γ-GCS activity and GSH level in HepG2 cells by caffeic acid obtained from PLE. Our results suggest that caffeic acid may be a key factor in the chemopreventive potential of perilla leaf components by increasing *de novo* synthesis of GSH.

1. Introduction

It is well recognised that oxidative and free-radical-mediated reactions contribute to aging and related diseases, such as cancer, coronary disease, and neurogenerative disorders (Smith et al., 1996; Weinbrenner et al., 2003). Endogenous or dietary factors play an important role in the antioxidative defenses of organisms against the reactive oxygen species (ROS) generated during normal cellular aerobic respiration (Kohen & Nyska, 2002). Current epidemiological data support that increased intakes of dietary antioxidants may help the tipping of the balance towards a proper antioxidant status (Halliwell, Murcia, Chirico, & Aruoma, 1995).

As a candidate dietary source, our research group has focused on *Perilla* [*Perilla frutescens* (L.) Britt. var. *japonica* (Hassk.) Hara]. This plant is an annual herbaceous plant native to Southeast Asian countries. Its leaves are often used in sushi, garnishes, and soups, and to wrap and eat cooked foods. The antioxidative, anti-allergic, anti-inflammatory, and anti-tumor promoting substances contained in perilla plants have earned considerable attention (Banno et al., 2004; Kim et al., 2007; Makino et al., 2003; Ueda, Yamazaki, & Yamazaki, 2002, 2003). Our previous *in vivo* study showed a protective effect of aqueous perilla leaf extract (PLE) on *tert*-butyl hydroperoxide (*t*-BHP)-induced oxidative hepatotoxicity (Kim et al., 2007). While the administration of *t*-BHP significantly decreased glutathione (GSH) level, the pretreatment of PLE remarkably increased GSH to an even higher level compared to that of the untreated control. Also, we observed a significant increase in hepatic γ-glutamylcysteine synthetase (γ-GCS) activity by the PLE treatment.

GSH is a ubiquitous molecule that plays an important role in intracellular free radical metabolism as well as xenobiotic detoxification (Biaglow et al., 1989). The enzyme catalysing the first and rate-limiting step in *de novo* GSH synthesis is γ-GCS (Dringen, 2000). To the best of our knowledge, the active component of PLE that is responsible for enhancing hepatic γ-GCS activity and GSH concentration has not been identified. Therefore, this research aimed to identify the functional compound of aqueous PLE possessing the aforementioned activities using the human hepatocellular cell line HepG2.

Abbreviations: γ-GCS, γ-glutamylcysteine synthetase; GSH, glutathione; PLE, perilla leaf extract; *t*-BHP, *tert*-butyl hydroperoxide; MEM, minimum essential medium; FBS, fetal bovine serum; MTT, 3-[4,5-dimethylthiazol-2-yl]-2,5-dephenyl tetrazolium; ATP, adenosine 5′-triphosphate; PEP, phospho(enol)pyruvate; NADH, β-nicotinamide adenine dinucleotide reduced dipotassium salt; BSA, bovine serum albumin; EtOAc, ethyl acetate; BuOH, *n*-butanol; MeOH, methanol; BSO, buthionine sulfoxide.

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2. Materials and methods

2.1. Chemicals

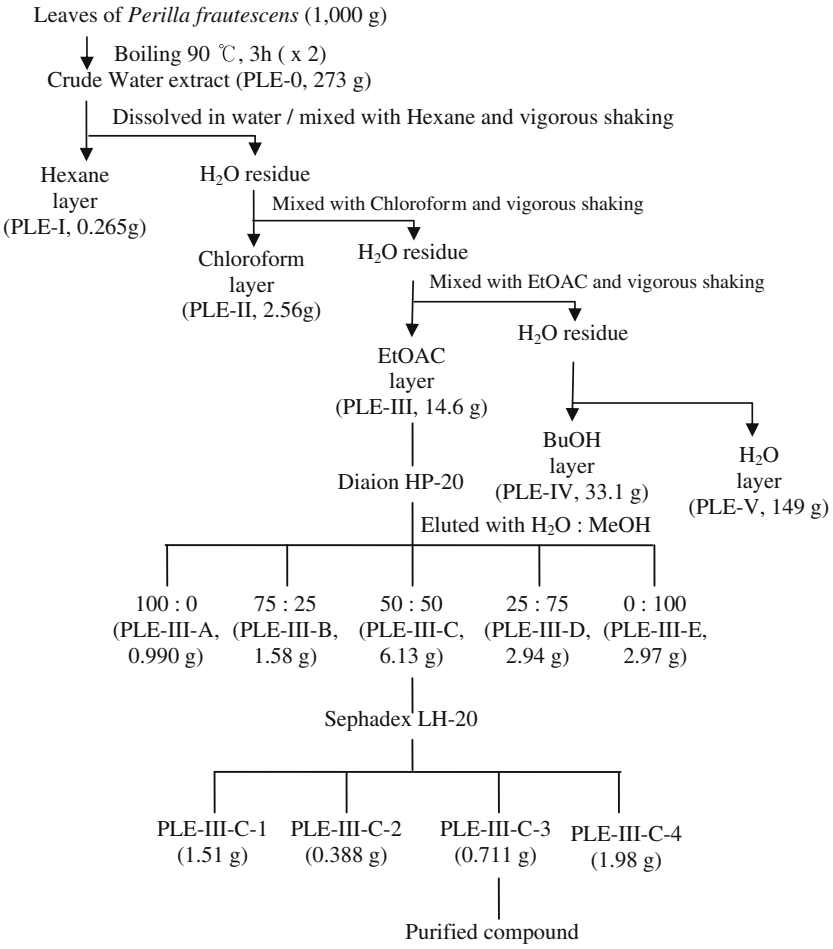
Minimum essential medium (MEM) and fetal bovine serum (FBS) were purchased from GIBCO® Life Technologies (Carlsbad, CA, USA). 3-[4,5-Dimethylthiazol-2-yl]-2,5-dephenyl tetrazolium (MTT), streptomycin, penicillin, adenosine 5′-triphosphate (ATP), phospho(enol)pyruvate (PEP), $MgCl_2$, L-glutamate, L-α-aminobutylate, β-nicotinamide adenine dinucleotide reduced dipotassium salt (NADH), pyruvate kinase, lactate dehydrogenase, and bovine serum albumin (BSA) were purchased from Sigma (St. Louis, MO, USA). Diaion HP-20 and Sephadex LH-20 were purchased from Mitsubishi Chemical Co. (Tokyo, Japan) and Amersham Biosciences (Uppsala, Sweden), respectively. The other chemicals were of the highest grade from commercial sources.

2.2. Plant materials

The edible leaves of a green type of perilla were purchased locally (Kyungdong Herb-Market, Seoul, Korea) and identified by Dr. B.W. Kang (College of Life Sciences and Biotechnology, Korea University). The fresh leaves were washed, and then blanched at 100 °C for 5 min, and immediately dried in a freeze dryer.

2.3. Extraction and isolation preparations

The dried perilla leaves (1.0 kg) were ground in a mortar and then soaked in distilled water (1 g/10 ml), followed by refluxing twice at 100 °C for 3 h, and then cooled. The undissolved remains were removed by filtration through a Whatman 41 filter paper (Clifton, NJ, USA) followed by a membrane filter with a 0.45 μm pore size (Millipore, Billerica, MA, USA). The filtrate was concentrated with a rotary vacuum evaporator (N-1000S, EYELA, Tokyo, Japan), and then lyophilised yielding a dried residue. For further fractionation of the dried PLE (273 g), the extract was resuspended in H_2O, and then extracted successively with *n*-hexane, chloroform, ethyl acetate (EtOAc), and *n*-butanol (BuOH) (Scheme 1). The yields of the *n*-hexane (PLE-I), chloroform (PLE-II), EtOAc (PLE-III), *n*-BuOH (PLE-IV), and water residue (PLE-V) extracts were 0.0971%, 0.938%, 5.35%, 12.1%, and 54.6%, respectively. The EtOAc-soluble portion (14.6 g) was applied to a Diaion™ HP-20 (250 × 45 mm; Mitsubishi Chemical Co., Tokyo, Japan) column, and then eluted by a gradient with increasing methanol (MeOH) in H_2O (100/0, 75/25, 50/50, 25/75, and 0/100, v/v; 237 ml of each eluent). The active fraction (PLE-III-C) eluted by the H_2O and MeOH (50/50, v/v) was further purified on a Sephadex™ LH-20 (Amersham Biosciences, Uppsala, Sweden) column (400 × 30 mm) in which the column was eluted with MeOH–H_2O (7:3) at 0.3 ml/min, and 3 ml/tube was collected. Finally, the active compound from fraction (PLE-III-C-3) was obtained by SymmetryPrep C18 (300 × 7.8 mm, 7 μm; Waters, Milford, MA, USA) column chromatography, in which elution was affected using a linear gradient of solvent mixtures of H_2O (solvent A) and MeOH (solvent B). The composition of B was held to 50% for 10 min, increased to 55% over 40 min and held for 10 min, and then returned to the initial concentration over 5 min at 1 ml/min. The eluted fraction was monitored at 320 nm.



Scheme 1. Isolation and purification procedure for *P. frutescens*.