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Research article

A brief method for preparation of gintonin-enriched fraction from ginseng

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ABSTRACT

Background: Ginseng has been used as a tonic for invigoration of the human body. In a previous report, we identified a novel candidate responsible for the tonic role of ginseng, designated gintonin. Gintonin induces $[Ca^{2+}]_i$ transient in animal cells via lysophosphatidic acid receptor activation. Gintonin-mediated $[Ca^{2+}]_i$ transient is linked to anti-Alzheimer's activity in transgenic Alzheimer's disease animal model. The previous method for gintonin preparation included multiple steps. The aim of this study is to develop a simple method of gintonin fraction with a high yield.

Methods: We developed a brief method to obtain gintonin using ethanol and water. We extracted ginseng with fermentation ethanol and fractionated the extract with water to obtain water-soluble and water-insoluble fractions. The water-insoluble precipitate, rather than the water-soluble supernatant, induced a large $[Ca^{2+}]_i$ transient in primary astrocytes. We designated this fraction as gintonin-enriched fraction (GEF).

Results: The yield of GEF was approximately 6-fold higher than that obtained in the previous gintonin preparation method. The apparent molecular weight of GEF, determined using sodium dodecyl sulfate-polyacrylamide gel electrophoresis, was equivalent to that obtained in the previous gintonin preparation method. GEF induced $[Ca^{2+}]_i$ transient in cortical astrocytes. The effective dose (ED_{50}) was 0.3 ± 0.09 μg/mL. GEF used the same signal transduction pathway as gintonin during $[Ca^{2+}]_i$ transient induction in mouse cortical astrocytes.

Conclusion: Because GEF can be prepared through water precipitation of ginseng ethanol extract and is easily reproducible with high yield, it could be commercially utilized for the development of gintonin-derived functional health food and natural medicine.

1. Introduction

Ginseng, the root of *Panax ginseng* C.A. Meyer, has been used as a tonic for human vitality and health [1]. Recent reports have shown that ginseng contains a novel G protein-coupled lysophosphatidic acid (LPA) receptor ligand, gintonin, in addition to ginsenosides. The primary action of gintonin is to induce $[Ca^{2+}]_i$ transient through LPA receptor activation, with a high affinity in cells

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expressing LPA receptors either endogenously or heterologously. Gintonin-mediated LPA receptor activation is coupled to diverse downstream events, including stimulation of phospholipase C, protein kinase C, mitogen-activated protein kinases, and phosphoinositide 4-kinase, through multiple G proteins such as $G\alpha_{i/o}$, $G\alpha_{12/13}$, and $G\alpha_{q/11}$ [2]. The transient elevation of intracellular Ca^{2+} via LPA receptor activation is a key mediator of diverse gintonin-mediated *in vitro* and *in vivo* effects. For example, gintonin regulates various Ca^{2+}-dependent ion channels [3–5] and modulates *N*-methyl-D-aspartic acid and $P2X_1$ receptors via Ca^{2+}-dependent signaling pathways [6,7]. Gintonin-mediated ion channels and receptor regulations are linked to the increase of gastrointestinal contractility by stimulation of pacemaker activity in the gastrointestinal system [8] and enhancement of synaptic transmission in hippocampal slices in the brain [9]. *In vivo* studies showed that gintonin reduces brain inflammation and amyloid plaque formation in transgenic Alzheimer's disease animal models and shows antimetastatic effect [10,11].

The previous methods for gintonin preparation included multiple steps using various organic solvents and anion exchange chromatography with a time-consuming separation process [12,13]. In addition, if gintonin has to be commercially utilized as a ginseng-derived functional health food, these previous processes for gintonin preparation are required for its *in vivo* safety test. In the present study, we developed a simple method for gintonin-enriched fraction (GEF) preparation using only ethanol and water from ginseng. We report here that this procedure simplified the GEF preparation process and produced a much higher yield of gintonin than the previous method. GEF induces $[Ca^{2+}]_i$ transient through the same signal transduction pathways as gintonin via LPA receptor activation in cultured mouse cortical astrocytes. Finally, the present report discusses the possibility of substituting anion exchange chromatography with water for GEF preparation.

2. Materials and methods

2.1. Materials

Four-year-old Korean white ginseng (Korea Ginseng Cooperation, Daejon, Korea) was purchased from a local ginseng market; the other reagents were purchased from Sigma-Aldrich (St. Louis, MO, USA).

2.2. Preparation of GEF from ginseng root and ginsenoside determination

One kilogram of 4-yr-old ginseng was ground into small pieces (> 3 mm) and refluxed with 70% fermentation ethanol eight times for 8 h at 80°C each. The ethanol extracts (150 g) were concentrated as described in Fig. 1B. Ethanol extract was dissolved in distilled cold water in a ratio of 1:10 and stored at 4°C in a cold chamber for 24 h. The supernatant and precipitate produced by water fractionation, after the ethanol extraction of ginseng, was separated by centrifugation (1977 *g*, 20 min). The precipitate was lyophilized after being centrifuged. This fraction was designated GEF with a yield of 1.3% (Fig. 1C). The representative ginsenosides, such as ginsenoside Rb1 and Rg1, were determined in the ethanol extract, supernatant, and precipitate from water fractionation by the Gyeonggi Bio Center (Suwon, Korea).

2.3. Gel filtration chromatography

Gel filtration chromatography of the ginseng ethanol extract, supernatant from water fractionation, or GEF was performed using an Superdex 75 column (10 × 300 mm) equilibrated with Tris–HCl (pH 8.0) on the BioLogic DuoFlow chromatography systems (Bio-Rad, Hercules, CA, USA) according to a previous report [12,13]. Fractions were collected with a flow rate of 0.5 mL/min and monitored at 280 nm. Each fraction was tested for $[Ca^{2+}]_i$ transient in cultured mouse cortical primary astrocytes [14].

2.4. Electrophoresis and Sudan Black staining

Ginseng ethanol extract, the supernatant from water fractionation, and GEF from ginseng were subjected to 12% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) independently [12]. The ethanol extract, supernatant from water fractionation, GEF, and gintonin (200 μg each) were loaded into individual lanes. After electrophoresis, the GEF bands were visualized by Coomassie Brilliant Blue R-250 staining [12]. The gel was stained with Sudan Black B solution producing 500 mg Sudan Black B (Sigma-Aldrich) in 20 mL acetone; 15 mL acetic acid and 85 mL water were added to the solution, stirred for 30 min, and centrifuged to remove the precipitate. The gel was stained in a solution overnight. The gel was destained with three changes in the following solution: 150 mL of acetic acid, 200 mL of acetone, and 650 mL of water [13].

2.5. Gintonin amino acid composition analysis

GEF (300 μg) from ginseng was hydrolyzed *in vacuo* with 6N HCl for 24 h at 110°C for general amino acid analysis. For the analysis of cysteine, GEF was hydrolyzed with 6N HCl for 24 h at 110°C after peroxidation treatment with formic acid, hydrogen peroxide (10:1). For the analysis of tryptophan, the sample was hydrolyzed with 4M methanesulfonic acid, and 4M KOH was added. Amino acids converted to phenyl isothiocyanate derivatives were analyzed with high-performance liquid chromatography (Hewlett Packard 1100 series; Hewlett Packard, Palo Alto, CA, USA) with a Waters Nova-Pak C18 column (3.9 mm × 300 mm) at the Korea Basic Science Institute (Seoul, Korea). Protein contents were determined using the Bradford method with bovine serum albumin as a standard [12].

2.6. Carbohydrate composition

GEF from ginseng was hydrolyzed in 2M trifluoroacetic acid for 4 h at 100°C for neutral sugar and hydrolyzed in 6N HCl for 4 h at 100°C for amino sugar and acid sugar in glass. Carbohydrate compositions of gintonin were analyzed using a high-performance anion exchange chromatography-pulsed amperometric detection system (HPAEC–PAD system; Dionex, Sunnyvale, CA, USA) with a CarboPac PA1 column at the Carbohydrate Bioproduct Research Center, Sejong University (Seoul, Korea). The molar ratios of monosaccharides were calculated from the peak areas. The carbohydrate contents were also determined using the phenol–sulfuric acid method for neutral sugar [12] and the anthrone method for acid sugar [12].

2.7. Lipid composition analysis

Total lipids in the ethanol extract, supernatant, and precipitate from water fractionation were determined using the procedure described by Folch et al [15]. The lipid composition of GEF was analyzed by hydrolyzing GEF from ginseng with 6N HCl for 4 h at 100°C or by digesting lipoprotein lipase to confirm lipid and hydrophobic moiety. Acid hydrolyzed or digested GEF was partitioned between distilled water and *n*-butanol (BuOH). The *n*-BuOH layer, after concentration, was partitioned further between distilled water and *n*-hexane. The *n*-hexane layer was prepared for lipid and