Isolation and Identification of an Autophagy-inducing Compound from Raphani Semen

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Abstract – The autophagy-lysosomal pathway is an important protein degradation system, and its dysfunction has been implicated in a number of neurodegenerative diseases, including Parkinson’s disease. Raphani Semen, one of the herbs of Yeoldahanso-tang (YH), has neuroprotective effects via the autophagy pathway. The activity-guided method was used to isolate and identify the components of Raphani Semen. In this experiment, the total extract of Raphani Semen was partitioned to n-butanol, methylene chloride, and water fractions. Flow cytometry data showed that only the water fraction showed autophagy-inducing activity in vitro. Compounds 1 and 2 were isolated from this water fraction by preparative HPLC separation. The structures of compounds 1 and 2 were identified as stachyose and raffinose, respectively, by the analysis of various spectral data (1H NMR, 13C NMR, and MS) and comparisons with standard stachyose and raffinose. Of these two compounds, raffinose showed autophagy-inducing activity in PC12 cells through the mTOR pathway.

Keywords – Autophagy, Raffinose, Raphani semen, Raphanus sativa L., Cruciferae

Introduction

Parkinson's disease (PD) is a degenerative disorder of the central nervous system. While the initial cause is undetermined, PD results from the death of the dopaminergic neurons of the substantia nigra, which is a region of the midbrain. Insufficient protein degradation, caused by various factors, may lead to the dopaminergic neuronal cell death that is related to PD and other neurodegenerative diseases (Cho, 2012).

The autophagy-lysosome pathway (ALP) is a mechanism that cleans the misfolded proteins and organelles in the cell (Ciechanover, 2005; Rubinsztein, 2006; Kim et al., 2012). “Autophagy”, literally meaning “self-eating”, describes a catabolic process in which cell constituents such as organelles and proteins are delivered to the lysosomal compartment for degradation (Nedelsky et al., 2008). Autophagy plays an important role in Parkinson’s disease. Microtubule-associated protein light chain 3 (LC3), a mammalian homologue of autophagy-related gene 8 (Atg8) in yeast, is recruited to the autophagosome membrane during autophagy and is considered a specific marker of autophagy (Kabeya et al., 2000).

Rapamycin, which is a lipophilic and macrolide antibiotic, induces autophagy by inactivating the protein mammalian target of rapamycin (mTOR), and as such, it is an autophagy enhancer (Berger et al., 2006). Several studies have shown that rapamycin, acting through the mTOR pathway, is neuroprotective in various neurological diseases (Erlich et al., 2007; Parker et al., 2000; Wu et al., 2008; Zemke et al., 2007). In the present study, we used rapamycin (200 nM) as a positive control in experiments to examine autophagy induction (Pan et al., 2008).

A previous study showed that Yeoldahanso-tang (YH), a Chinese herbal medicine, had neuroprotective effects via autophagy enhancement in Parkinsonian model systems, both in vivo and in vitro (Bae et al., 2011). Raphani Semen is one of the herbs of YH, and it was chosen for further study because its extract showed autophagy enhancement in PC12 cells.

Raphani Semen belongs to the Cruciferae, meaning “cross-bearing,” family of flowering plants (Angiosperms), which contains over 330 genera and approximately 3,700 species. The largest genera are Draba (365 species), Cardamine (200 species, although the definition of this genus is controversial), Erysimum (225 species), Lepidium
The family contains well-known species such as *Brassica oleracea* (broccoli, cabbage, cauliflower, etc.), *Brassica rapa* (turnip, Chinese cabbage, etc.), *Brassica napus* (rapeseed, etc.), *Raphanus sativus* (common radish), *Armoracia rusticana* (horseradish), *Matthiola* (stock), *Arabidopsis thaliana* (model organism) and many others. The family is cosmopolitan but is concentrated in the northern temperate regions and reaches its maximum diversity around the Mediterranean area.

**Experimental**

**Plant material** – In this study, *Raphani Semen* (RS) was provided by the Botany and Drug Department of the Oriental Hospital of Daejeon University (Daejeon, Korea), according to the Korean herbal pharmacopoeia (The Korea Food and Drug Administration, 2002).

**Chemicals and instruments** – Monodansylcadaverine (MDC), Raffinose, stachyose and rapamycin were purchased from Sigma-Aldrich (Saint Louis, MO, USA). NMR experiments were performed on a Varian NMR System (Unity Plus 500 MHz). Low resolution ESI-MS (electrospray ionization mass spectrometry) was measured on an Agilent Technologies 1200 series HPLC system using a 6120 Quadrupole. An YL9100 HPLC (YL instrument Co., Ltd, Anyang, South Korea) system and YMC-ODS-AQ column (150 × 1.6 mL, 5 µm YMC, Tokyo, Japan) were used for analysis. Semi-preparative HPLC 321 Pumps (Gilson Inc, Middleton, WI, USA) and Delta-Pak C18 column (300 × 30.00 mm, 15 microns, Waters, Tokyo, Japan) were used to isolate compounds. In bioassay experiments, microplate reader (BIO-TEKR, Dower Wave XS, Winooski, VT, USA) was used to measure cell viability. Western immunoblotting assay used LAS-4000 Luminescent Image Analyzer (Fujifilm, Tokyo, Japan). Flow cytometry (BD FACS Calibur, San Jose, CA, USA) was used to analyze MDC staining cells.

![Extraction scheme for the isolation of active compound from Raphani Semen.](image-url)