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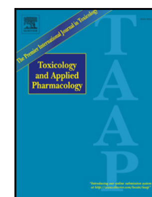

A silk peptide fraction restores cognitive function in AF64A-induced Alzheimer disease model rats by increasing expression of choline acetyltransferase gene

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ABSTRACT

This study investigated the effects of a silk peptide fraction obtained by incubating silk proteins with Protease N and Neutrase (SP-NN) on cognitive dysfunction of Alzheimer disease model rats. In order to elucidate underlying mechanisms, the effect of SP-NN on the expression of choline acetyltransferase (ChAT) mRNA was assessed in F3.ChAT neural stem cells and Neuro2a neuroblastoma cells; active amino acid sequence was identified using HPLC-MS. The expression of ChAT mRNA in F3.ChAT cells increased by 3.79-fold of the control level by treatment with SP-NN fraction. The active peptide in SP-NN was identified as tyrosine-glycine with 238.1 of molecular weight. Male rats were orally administered with SP-NN (50 or 300 mg/kg) and challenged with a cholinotoxin AF64A. As a result of brain injury and decreased brain acetylcholine level, AF64A induced astrocytic activation, resulting in impairment of learning and memory function. Treatment with SP-NN exerted recovering activities on acetylcholine depletion and brain injury, as well as cognitive deficit induced by AF64A. The results indicate that, in addition to a neuroprotective activity, the SP-NN preparation restores cognitive function of Alzheimer disease model rats by increasing the release of acetylcholine.

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1. Introduction

Not only aging, but also central nervous system (CNS) diseases, such as Alzheimer disease (AD), one of the most devastating neurodegenerative diseases, progressively reduce brain functions. Impairment of cognitive functions characterized by learning and memory loss is a specific feature in aging and AD patients (Terry and Davies, 1980). Degeneration of presynaptic cholinergic system is a major cause of cognitive deficit in AD patients (Coyle et al., 1983; Whitehouse et al., 1982) and is known to be attributable to the decreased activity of choline acetyltransferase (ChAT), which is necessary for acetylcholine (ACh) synthesis (Kasa et al., 1997; Terry and Buccafusco, 2003).

AF64A is a choline analog that is taken up only by the high-affinity choline transport system into cholinergic neurons (Fisher et al., 1982) and causes alterations in mRNA expression and activity of ChAT (Bessho et al., 2008; Fan and Hanin, 1999; Yamazaki et al., 1992). Therefore, AF64A administration was found to decrease the release of ACh leading to cognitive impairments, including memory and learning deficits, a salient characteristic of AD (Abe et al., 1993; Bessho et al., 2008; Yamazaki et al., 1992).

Thus far, AD therapy has relied on compounds that increase the ACh concentration, such as acetylcholinesterase (AChE) inhibitors, as well as *N*-methyl-D-aspartate (NMDA) receptor antagonists (Bessho et al., 2008; Eleti, 2016; Musial et al., 2007; Takashina et al., 2008; Terry and Buccafusco, 2003). In addition, enhancers of mRNA expression of ACh-

Abbreviations: ACh, acetylcholine; AChE, acetylcholinesterase; AD, Alzheimer disease; BF-7, brain factor-7; ChAT, choline acetyltransferase; CNS, central nervous system; CSF, cerebrospinal fluid; F3.ChAT, neural stem cell encoding ChAT gene; GFAP, glial fibrillary acidic protein; MAO-B, monoamine oxidase-B; NSC, neural stem cell; PD, Parkinson disease; SAA, silk amino acid; SP-NN, silk protein degraded by Protease N and Neutrase; SP-PN, silk protein degraded by Protease P and Neutrase; SP, silk peptide; SPPS, solid phase peptide synthesis.

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synthesizing enzyme ChAT were used to recover impaired learning and memory functions (Egashira et al., 2003; Karakida et al., 2007; Shin et al., 2016; Wang et al., 2000). In particular, ginsenoside Rb1 was used to increase the expression of ChAT mRNA (Qi et al., 2011), thereby increasing synaptosomal choline uptake and the ACh release (Benishin, 1992; Benishin et al., 1991; Scholey et al., 2010). In addition, in our previous studies, we demonstrated that transplantation of neural stem cells (NSCs) encoding ChAT gene (F3.ChAT) markedly recovered cognitive function of animal models of AD and aging (Park et al., 2012a, 2013).

Silk proteins from silkworm (*Bombyx mori*) are well known to have pharmacological activities such as anti-diabetic effect (Lee et al., 2007; Park et al., 2002). When degraded by enzymes, silk proteins yield specific sizes or compositions of silk peptides (SPs) that show diverse bioactivities, including anti-diabetic, hypocholesterolemic, and antioxidative actions (Kato et al., 1998; Kim et al., 2008; Lee et al., 2007; Zhaorigetu et al., 2003). In addition, silk amino acids (SAAs; composed of 19 amino acids) obtained from full degradation of silk proteins via acidic (HCl) hydrolysis have been reported to prevent oxidative injures of tissues, thereby enhancing physical stamina (Shin et al., 2009a, 2009b). Another study reported that SAA preserved dopaminergic nerves in the Parkinson disease (PD) model animals to improve the animals' movement disorders (Kim et al., 2011). Still another study reported that SP improved cognitive function of rats with aging brain facilitated by D-galactose (Park et al., 2011).

Such results led us to investigate the cognition-enhancing effect of an enzyme-degraded silk peptide prepared via the incubation process of silk proteins with Protease N and Neutrase (SP-NN) in AD model animals. In order to elucidate the underlying mechanisms, the effect of SP-NN on the expression of ChAT mRNA was assessed in F3.ChAT neural stem cells and the active amino acid sequence was identified using HPLC-MS.

2. Materials and methods

2.1. SP preparation

Freeze-dried silk peptide-NN (SP-NN; Worldway code: PF-B6) was obtained from Worldway Co., Ltd. (Sejong, Korea). To prepare SP-NN, silk proteins were incubated for 12 h with Protease N (150,000 U/g; Amino G from *Bacillus subtilis*) plus Neutrase (160,000 U/g; *Bacillus amyloliquefaciens*). In short, the cocoon were dissolved at 115 °C for 60 min in the KCl solution and then sterilized at 90 °C for 30 min. After being cooled to 50 °C, the proteins were degraded using the above enzymes at pH 5.8 and autoclaved at 90 °C for 30 min to inactivate the enzymes. Through the GPC-RI analyses, the peak molecular weight of SP-NN was identified as 962 (Park et al., 2011). Freeze-dried SP-NN was reconstituted with distilled water at the concentration of 200 mg/ml and then centrifuged at 3000 rpm for 5 min. The supernatant was used for gel filtration chromatography and assay for ChAT mRNA expression.

2.2. Expression of ChAT mRNA

The effects of SP-NN on neuronal ChAT mRNA expression were evaluated using human NSC encoding ChAT gene (F3.ChAT) (Park et al., 2011, 2012a–b, 2013; Shin et al., 2016) and Neuro2a murine neuroblastoma cells (Misawa et al., 1994). F3.ChAT and Neuro2a cells (1×10^6 cells/ml) were incubated with 1, 10 or 100 μg/ml of SP-NN at 37 °C for 24 h in a 5% CO_2 incubator. Total RNA was extracted from F3.ChAT cultures using the Trizol method (Invitrogen, Carlsbad, CA, USA) for the reverse transcriptase-polymerase chain reaction (RT-PCR) analysis of ChAT mRNA expression. Complimentary DNA templates from each sample were prepared from 1 μg of total RNA primed with oligo dT primers using 400 U of Moloney Murine Leukemia Virus reverse transcriptase (Promega, Madison, WI, USA) followed by 30 PCR cycles. The RT-PCR products were separated electrophoretically on 1.2% agarose gel containing ethidium bromide and then visualized under the UV light. The primers used for the RT-PCR of human or mouse ChAT are as follows: human sense: 5′-CTCTGACCTGTCAGAAGAAT-3′, antisense: 5′-GACGCTGACACTTACAGAAT-3′, and mouse sense: 5′-GTGGCCCAGAAGAGCAGTAT-3′, antisense: GGACTCCCTGGACATGATCG-3′.

2.3. Separation and purification

The supernatant of SP-NN solution was separated on a Sephadex G-15 (G15–150, MWCO: <1500 Da, Sigma-Aldrich, MO, USA) gel filtration chromatography column (φ1.5 × 120 cm, Pyrex, New York, NY, USA), eluted with distilled water at the flow of 0.3 ml/min using a HPLC pump (Waters 510, Milford, MS, USA) and then collected in a 5 ml/tube using a fraction collector (2110, BIO-RAD, Hercules, CA, USA). Elution curves were obtained by measuring absorbance at 280 nm using a spectrophotometer (UV-1650 PC, Shimadzu, Tokyo, Japan). The fractions showing similar absorbance at 280 nm were collected and then dried with a rotary evaporator (N-1000, Eyela, Tokyo, Japan) and then evaluated for ChAT mRNA expression activity.

The fractions showing high ChAT mRNA expression activity were resolved in distilled water and then separated on a semi-preparative HPLC (SP930D, Younglin Instrument, Anyang, Gyeonggi, Korea) equipped with HECTOR-M (250 × 21.2 mm, RStech Co., Daejeon, Korea) at the flow rate of 5.0 ml/min. The mobile phase was water/acetonitrile/TFA (85/15/0.1, v/v/v), the injection volume of sample was 100 μl, and the detector was an UV-detector (Younglin Instrument) at 280 nm.

The fractions separated by semi-preparative HPLC were purified by a preparative recycling HPLC system (JAI LC-9201 model, JAI, Tokyo, Japan) equipped with a JAI GEL-GS310 column (500 × 20 mm, φ5 μm, JAI). The mobile phase was HPLC grade water, the injection volume of sample was 2000 μl, and the detector was an UV-detector (Younglin Instrument) at 280 nm.

2.4. Mass spectrometry analysis

MS and MS/MS measurements of the active compounds were performed in the positive ion mode using LC-ESI-MS and TOF-MS/MS, respectively (Hwang et al., 2011). The LC analysis was performed on the Agilent 1100 series instrument (Agilent Technologies, Santa Clara, CA, USA) equipped with a ZORBAX 300SB_C18 (1 × 150 mm, φ3.5 μm, Agilent Technologies). The MS analysis was performed on the Hybrid Quadrupole TOF LC/MS/MS Mass Spectrometer (AB Sciex Instrument, Redwood City, CA, USA) using the ESI mode. The scan range was from 200 to 2000 MHz, scan rate was 1 s/scan, and the source temperature was 120 °C. The ^{1}H nuclear magnetic resonance (NMR, 500 MHz), Two Dimension Heteronuclear Multiple Bond Correlation (2D-HMBC) spectra, ^{13}C NMR (900 MHz), and Distortionless Enhancement by Polarization Transfer (DEPT) spectra were recorded on a spectrometer (Advance 500 spectrometer, Bruker Analytic GmbH, Karlsruhe, Germany) using D_2O as a solvent.

2.5. Synthesis of standard compound

The Trt-Cl resins and Fmoc (fluorenylmethyloxycarbonyl)-derivatized tyrosine and glycine were procured from GL Biochem (Shanghai, China); coupling and cleavage cocktail reagents were from Alfa Aeser (Ward Hill, MA, USA). Tyrosine-glycine was synthesized by Fmoc solid phase peptide synthesis (SPPS) procedures using ASP48S (Peptron, Daejeon, Korea) and purified by the reverse phase HPLC (Shimadzu Prominence HPLC; Shimadzu, Tokyo, Japan) using a Vydac Everest C18 column (250 × 22 mm, 10 μm; Grace, Columbia, MD, USA). Elution was performed with a water-acetonitrile linear gradient [10– 75% (v/v) of acetonitrile] containing 0.1% (v/v) of trifluoroacetic acid. Molecular weights of the purified peptide were confirmed using LC/MS (Agilent HP1100 series; Agilent, Santa Clara, CA, USA) and then

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