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Identification of antioxidative peptides from defatted walnut meal hydrolysate with potential for improving learning and memory

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ABSTRACT

Defatted walnut meal (DWM), a main byproduct of walnut oil production, is rich in proteins with a high essential amino acid content. Our research was focused on the functional activities of defatted walnut meal hydrolysate (DWMH). DWMH exhibited relatively strong hydroxyl scavenging and oxygen radical absorbance capacities, protective effect on H_2O_2-injured PC12 cells compared with GSH and cerebrolysin. Besides, DWMH could combat D-galactose induced learning and memory impairments in the Morris water maze test and in the Dark/light avoidance test of mice. Seventy-seven peptides were identified by UPLC-ESI-MS/MS in the most potent antioxidative fraction of DWMH. WSREEQEREE and ADIYTEEAGR were the peptides that most likely accounted for the suppression of H_2O_2-induced apoptosis in PC12 cells. The mixture of the commercially available amino acids did not offer any protection on H_2O_2-injured PC12 cells, suggesting that amino acid sequence rather than amino acid composition was the dominant influencing factor. DWMH could be recommended as natural antioxidants for the development of functional foods targeting memory impairments.

1. Introduction

The peptides possessing specific health functionality including those that combat oxidative stress, have attracted considerable research interest. The antioxidative peptides derived from soy protein (Park, Lee, Baek, & Lee, 2010), peanut protein (Ji, Sun, Zhao, Xiong, & Sun, 2014), hempseed protein (Lu et al., 2010), egg white protein (Liu, Jin, Lin, Jones, & Chen, 2015) and chickpea protein (Torres-Fuentes, Contreras, Recio, Alaiz, & Vioque, 2015) had exhibited strong radical scavenging activities. In addition, some studies had revealed that antioxidative peptides could improve learning and memory ability of the rats (Knafo et al., 2012). Pei et al. (2010) reported that the protective effect of marine collagen peptide against oxidative stress in the brain was probably involved in the mechanism of the impairment of learning and memory. However, the most effective approach and precise underlying mechanisms for memory-enhancing effects remained unknown. It was hypothesized that oxidative stress and free radical damage would contribute to memory deficits, and antioxidant substance could improve cerebral functions (Looi & Sachdev, 2003; Socci, Crandall, & Arendash, 1995). From recently, cerebrolysin is a compound with neurotrophic and neuroprotective, which prepared from porcine brain protein by protease hydrolysis, containing 75% free amino acids and 25% low molecular weight peptides (<10 kDa). It was reported that cerebrolysin could exhibit growth factor characteristics and promote the survival and sprouting of neurons (Hartbauer, Hutter-Paier, & Windisch, 2001; Schauer et al., 2006a). It is possible that antioxidative peptides with strong free radical-scavenging activity could scavenge the free radicals which damaged biological systems, and then exhibit some beneficial effects on age, stress or impairment-related memory degeneration.

Defatted walnut meal (DWM), as the main by-product obtained from the roasted kernels of walnut (*Juglans regia* L.) during oil extraction, is composed of more than 40% proteins with a high essential amino acid content (Sze-Tao & Sathe, 2000). While DWM is conventionally used as animal feed or fertilizer, there is increasing interest in exploring its protein components as natural antioxidants or neuroprotective agents. Chen, Yang, Sun, Niu, & Liu, (2012) identified an antioxidant peptide (Ala-Asp-Ala-Phe) in walnut protein hydrolysates which possesses an inhibitory effect on lipid peroxidation comparable to that of GSH. Furthermore, Liu et al. (2013) found an ACE inhibitory peptide (Trp-Pro-Glu-Arg-Pro-Pro-Gln-Ile-Pro) in walnut protein hydrolysate.

Antioxidative effects of a substance can be assessed using various in vitro methods, including in vitro chemical antioxidant capacity assays based on different mechanisms such as hydrogen-atom-transfer (HAT) reaction and single-electron-transfer (SET) reaction, and in vitro

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cultured cell model bioassays based on cytoprotective activity against H_2O_2-induced oxidative damage to rat adrenal pheochromocytoma (PC12) cells (Gu et al., 2012). Furthermore, the memory-enhancing activities of peptides could be validated in vivo using animal and/or human models. The Morris water maze test and Dark/light avoidance test are two commonly used in vivo tests for evaluating the effects of supplementary feeding/treatment on learning and memory ability in mice (Bourin & Hascoët, 2003; Brandeis, Brandys, & Yehuda, 1989). In the present study, the in vitro hydroxyl radical scavenging activity, oxygen radical absorbance capacity (ORAC), and protective effect on H_2O_2-induced damaged PC12 cells of defatted walnut meal hydrolysate (DWMH) were assessed. To examine whether or not an association between the memory-enhancing effect and antioxidant activity, the memory-enhancing effects of DWMH were also evaluated using the above-mentioned mouse tests. The bioactive peptides from DWMH that likely responsible for these effects were also proposed.

2. Materials and methods

2.1. Materials, chemicals and animal products

Defatted walnut meal was provided by Huizhiyuan Food Co., Ltd. (Lincang, China). Pancreatin (4.6×10^6 U/g) was purchased from Shisheng Science and Technology Co., Ltd. (Hangzhou, China). GSH, 2,2′-azobis(2-methylpropionamide)-dihydro-chloride (AAPH), fluorescein disodium (FL), 3-(4,5-dimethylthiazol-2-yl) 2,5-diphenyltetrazolium bromide (MTT), 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (Trolox) were purchased from Sigma-Aldrich Company (St. Louis, Missouri, USA). SP-825 macroporous adsorption resin was purchased from Sunresin New Materials Co., Ltd. (Xi'an, China).

The rat pheochromocytoma line 12 (PC12) cells were obtained from the Cell Institute of Biochemistry and Cell Biology (Shanghai, China). Dulbecco's modified Eagle's medium (DMEM), fetal bovine serum (FBS), penicillin and streptomycin were purchased from Gibco BRL, Life Technologies (New York, USA). Cerebrolysin was purchased from Ebewe Pharma Arzneimittel (Austria). 6-Carboxy-2′, 7′ dichlorofluorescein diacetate (DCFH-DA) and Annexin V-FITC Apoptosis Detection kit were purchased from Beyotime Institute of Biotechnology (Shanghai, China).

D-galactose was purchased from Hengxin Chemical-Regent Co., Ltd. (Shanghai, China). Piracetam was purchased from the South China Pharmaceutical Co., Ltd. (Dongguan, China). All other reagents for animal experiment were purchased from Ruiqi Biological Technology Co., Ltd. (Shanghai, China).

2.2. Preparation of DWMH

Defatted walnut meal protein (DWMP) was extracted following the method of (Mao, Hua, & Chen, 2014). The protein content of DWMP was $77.75 \pm 1.22\%$ (determined by Kjeldahl method, $N \times 5.3$, wet basis). The freeze-dried DWMP was used for the hydrolysis catalyzed by pancreatin at a substrate to enzyme ratio of 20:1 (*w*/*w*) under the optimal conditions (pH 8.0, 55 °C for 12 h). The hydrolysis was stopped thermally using a water bath (boiling water) for 10 min and cooled to room temperature, pH adjusted to 7.0, centrifuged at 8000 *rpm* and 4 °C for 15 min, then freeze-dried.

2.3. Determination of in vitro antioxidant activities of DWMH

The freeze dried DWMH was reconstituted with deionized water to the concentration of 1 mg/mL, prior to the in vitro antioxidant activity assay.

GSH is a natural antioxidative tripeptide that prevents damage to important cellular components caused by reactive oxygen species (Zheng et al., 2012). It was used as a positive control. Cerebrolysin consists of low molecular weight peptides with neuroprotective and neurotrophic properties similar to naturally occurring growth factors (Schauer et al., 2006a). It was used as a positive control in cells experiment.

2.3.1. The hydroxyl radical scavenging activity assay

The hydroxyl radical scavenging activity was determined by the method of (You, Zhao, Regenstein, & Ren, 2011).

2.3.2. The ORAC assay

The ORAC assay was determined by the method of (Zheng et al., 2012).

2.3.3. The viability of cultured PC12 cells and assay

The PC12 cells were grown in a humidified atmosphere (5% CO_2, 37 °C), using DMEM supplemented with 10% (*v*/*v*) FBS, 100 U/ml of penicillin-streptomycin, and collected in the log phase. To determine the radical-induced cytotoxicity, cells were seeded in a 96-well plate (5×10^3 cells/well) for 24 h and treated with various concentrations (0.10, 0.25, 0.50 mg/mL) of DWMH for a further 24 h. Cellular oxidation was induced by exposing the cells to a H_2O_2 solution (0.40 mM; which was fleshly prepared and stored at 4 °C before use) for 90 min. The H_2O_2-induced damage was halted by removing the H_2O_2-containing medium and replacing it with the fresh medium. Cell viability was determined using the MTT method (Gu et al., 2012). For comparison purposes, the aqueous solutions of the commercially available amino acids (termed as "AA-mixture") that resemble the major amino acid constituents of DWMH and cerebrolysin were used instead of DWMH for the above cell-based assays.

2.3.4. Assessment of the intracellular ROS

The accumulation of the intracellular ROS was determined by the fluorescent probe DCFH-DA following the method of (Muthaiyah, Essa, Chauhan, & Chauhan, 2011) with some modifications. PC12 cells were seeded in a 6-well plate (2×10^4 cells/well) and cultured for 24 h, treated with different concentrations of DWMH (0.10, 0.25, 0.50 mg/mL), and then incubated for a further 24 h. Cellular oxidation was induced by exposing the cells to the above-mentioned 0.40 mM H_2O_2 for 90 min before the medium was replaced with a fresh medium. The obtained cells were incubated with 10 μM DCFH-DA at 37 °C for 60 min in the dark, before being washed with phosphate buffered saline (PBS) to remove the extracellular DAFH-DA. The resultant cells were then suspended in PBS and subject to the fluorescence intensity measurement using a Quanta SC flow cytometer (Beckman Coulter, USA).

2.3.5. Assessment of apoptosis

The apoptotic cells were determined using the Annexin V-FITC/Propidium iodide (PI) apoptosis assay kit following the method of (Xiao et al., 2013) with minor modifications. The PC12 cells were seeded in a 6-well plate (2×10^4 cells/well) and cultured for 24 h, before being treated with DWMH (0.50 mg/mL) and incubated for a further 24 h. Cellular oxidation was induced by exposing the cells to the above-mentioned 0.40 mM H_2O_2 for 90 min, before the medium was replaced with a fresh medium. The cells were harvested, rinsed twice with PBS, and re-suspended in 100 μL of binding buffer with 2 μL of Annexin V-FITC. The mixture was incubated on the ice for 15 min, to which 400 μL of binding buffer and 1 μL of PI were added. The resultant cells were collected and subjected to immediate analysis using flow cytometer.

2.4. In vivo memory improvement effect of DWMH

All the in vivo tests were carried out by the School of Life Science of Sun. Yat-sen University (Guangzhou, China), under the permission from the university's ethics committee for performing all animal experiments. One hundred and fifty-six BALB/c mice (18–22 g, Approval No. SYXK 2009-0020) were purchased from Guangdong Medical Laboratory