Neuroprotective Effects of Soybean Oligopeptides (SOPs) Against H$_2$O$_2$-induced Oxidative Stress in PC12 Cells

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Abstract. In the study, soybean protein isolate was hydrolyzed with Alcalase, hydrolysates were separated by membrane ultrafiltration to obtain SOPs. We examined the antioxidant properties of SOPs, including Fe$^{2+}$ chelating capacity, free radical scavenging activities against 2,2-diphenyl-1-picrylhydrazyl radical (DPPH), oxygen radical absorbance capacity (ORAC), and inhibiting the autoxidation of linoleic acid capacity. Then the neuroprotective effect of SOPs against H$_2$O$_2$-induced lipid peroxidation and cell death in PC12 cells was evaluated. The PC12 cell line pretreated with different concentrations (0.1, 0.5, and 1 mg/mL) of the SOPs and then treated with H$_2$O$_2$ to induce lipid peroxidation and neurotoxicity. The neurotoxicity was assessed by cell viability, lactate dehydrogenase (LDH) release and morphological characteristics. The results indicated that SOPs exhibited potent antioxidant activities, and pretreatment of PC12 cells with SOPs, prior to H$_2$O$_2$ exposure, significantly increased the survival of cells, and reduced the levels of LDH. These findings suggest that SOPs can protect PC12 cells against H$_2$O$_2$-induced lipid peroxidation and cell death as a neuroprotective agent.

Keywords: neuroprotective, PC12 cells, Soybean oligopeptides, Oxidative stress

1. Introduction

The number of people suffering from neurological disorders has been increased worldwide, especially in the developed countries [1]. Oxidative stress as a result of aberrant production of reactive oxygen species (ROS) is the major culprit in neuronal cell degeneration observed in neurodegenerative diseases, such as Alzheimer’s disease, Parkinson’s disease [2]. The imbalance between oxidants and antioxidants leads to disruption of redox signaling leading to accumulation of ROS. Excessive generation of free radicals causes damage to all kinds of biomolecules, such as lipids, proteins and DNA, in addition, lipid peroxidation is the main nerve toxic [3]. Therefore, current attention has been dedicated to find natural neuroprotective agents that can reduce oxidative stress in neurons, might be an appropriate choice for the treatment of neurodegenerative diseases [4].

Soybean based food has a long lasting tradition, especially in Asia. Moreover, soybean protein is a potential dietary source of bioactive peptides, enzymatic hydrolysis of proteins is one effective approach that can be used to release various bioactive peptides [5]. The soybean protein-derived peptides have been reported to possess a wide variety of biological activities such as antihypertensive, antithrombotic, opioid, antiAmyloid and antioxidant activities. Previous studies have also shown that SOPs through Alcalase have relatively higher antioxidant activities and antiAmyloid effects [6], and indicate that SOPs may reduce oxidative stress in neurons as neuroprotective agents. However, the direct effect of SOPs on the neuroprotective efficacy has not been investigated. Accordingly, the present study aimed to evaluate the potential neuroprotective effect of SOPs on H$_2$O$_2$-induced oxidative stress and neurotoxicity and in PC12 cells.

2. Materials and methods

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2.1. Materials

The soybean protein isolate was obtained from Harbin Hi-Tech Soybean Food co., Ltd (Harbin China). Alcalase 2.4 L (2.4 AU/g) was purchased from Novo Nordisk (Bagsvaerd, Denmark). The lactate dehydrogenase (LDH) assay kits was purchased from Beyotime Institute of Biotechnology (Haimen, China). Dulbecco's Modified Eagle's Medium (DMEM) and Fetal bovine serum (FBS) was purchased from Gibco BRL, Life Technologies (USA). EDTA, DPPH, Fluorescein, AAPH, Trolox were purchased from Sigma-Aldrich (USA). MTS from Promega (Msdiso, USA) and other chemicals were purchased from Beijing Chemical Plant (Beijing, China).

2.2. Preparation of SOPs

Soybean protein isolate was dispersed in distilled water to obtain 10% protein slurry (w/v) was hydrolyzed in a 1000 mL reactor under the controlled temperature and pH. The mixture was heated to 90°C, for 10 min, in order to denature the protein. The pH value was adjusted to 8.0 using 1 mol/L NaOH and incubated in 55°C water bath. The protein hydrolysis was initiated by the addition of a 0.052 AU/g dosage of Alcalase based on protein content and stirred for 3 h. After the hydrolysis reaction was terminated by boiling for 10 min and the solutions were centrifuged at 10000 rpm for 15 min at 4°C to obtain soybean protein hydrolysates. The hydrolysates were ultra-filtered through a membrane with 1 kDa nominal molecular weight limit to obtain the soybean oligopeptides. The SOPs were freeze-dried and stored at -20°C before further analysis.

2.3. DPPH radical-scavenging activity

The DPPH radical-scavenging activity of SOPs was measured according to the previously reported method with some modifications [7]. An aliquot of 100 μL 0.1 mM DPPH (dissolved in 95% ethanol) was mixed with the same volume of sample solution. The mixture was shaken and left for 30 min at room temperature. The absorbance of the resulting solution was measured at 517 nm. The blank substituted 100 μL of buffer solution instead of the sample, and Trolox was used as a standard.

2.4. Metal ion-chelating activity

The ability of SOPs to chelate Fe²⁺ ions was evaluated according to the previously reported method with some modifications [8]. Briefly, 50 μL sample solution was mixed with 50 μL of 0.2 mM ferrous chloride solution. After 3 min the reaction was initiated by the addition of 200μL of 0.5 mM ferrozine. The mixture was shaken vigorously and left at room temperature for 10 min. Absorbance was measured at 522 nm to determine chelating activity using EDTA as a standard.

2.5. Measurement of oxygen radical absorbance capacity (ORAC)

The ORAC assay was conducted to kinetically measure the peroxyl radical scavenging activity of SOPs with Trolox as the antioxidant standard [9]. Fluorescein was used as the fluorescent probe and the peroxyl radicals were generated from AAPH in 75 mmol/L phosphate buffer (pH 7.4). Excitation and emission wavelengths were 485 and 530 nm, respectively. Trolox equivalents (TE) were calculated using the relative area under the curve for samples compared to a Trolox standard curve prepared under the same experimental conditions.

2.6. Inhibition of linoleic acid autoxidation

The lipid peroxidation inhibition activity of SOPs was measured in a linoleic acid model system according to the method of Osawa and Namiki [10] with some modifications. The sample dissolved in 5 mL of 50 mM phosphate buffer (pH 7.0) was added to a solution of 65 μL of linoleic acid and 5 mL of 99.5% ethanol. The total volume was then adjusted to 12.5 mL with distilled water. The mixture was incubated in at 60 ± 1 °C for 7 days in a dark room. The degree of oxidation of linoleic acid was measured using the ferric thiocyanate method. Briefly, 0.1mL of the reaction mixture was mixed with 4.7 mL of 75% ethanol, 0.1 mL of 300 g/L ammonium thiocyanate and 0.1 mL of 20 mM ferrous chloride solution in 35% HCl. After 3 min of incubation the colour development, which represents linoleic acid oxidation, was measured at 500 nm.

2.7. Cell culture
The PC12 cell line was obtained from the Institute of Biochemistry and Cell Biology, SIBS, CAS (Shanghai, China). Cells cultured in DMEM supplemented with 10% FBS, 100 units/mL penicillin and 100 μg/mL streptomycin in an incubator aerated with 5% CO₂ at 37 °C.

2.8. Analysis of cell survival

The metabolic status of the mitochondria of PC12 cells was analyzed by the MTS assay. The cells were seeded (5x10⁴ cells/800 μL/well) and cultured in 24-well plates. The cells were then pre-incubated with SOPs for 24 h followed by incubation with or without 200 μM H₂O₂ for 12 h. After that, 100 μL of MTS (0.5 mg/mL) was added to each well and then incubated for 1h at 37 °C, the absorbance was determined at 490 nm using a microplate reader. The value for cell viability was converted to the percentage of control culture value. The morphology of the cells was also observed and photographed under a microscope.

2.9. LDH leakage

The LDH leakage was detected with an assay kit (Beyotime, Haimen, China) according to the manufacturer’s protocol. The culture medium was collected and retained for LDH determination. The adherent cells were washed twice with PBS and then lysed by the cell lysis buffer to release the intracellular LDH of the living cells into the new supernatant. After the reaction, each sample was measured at wavelength of 450 nm. LDH leakage was calculated as the ratio of released LDH to total LDH.

2.10. Statistical analysis

The results were expressed as means ± SD. Differences among different experimental groups were tested for significance using one-way analysis of variance, taking **P < 0.01 as significant.

3. Results and Discussion

3.1. Antioxidant capacity of SOPs

The antioxidant activities of SOPs were estimated by various antioxidant methods, including DPPH scavenging, Fe²⁺ chelating, ORAC and inhibition of linoleic acid autoxidation assays. As shown in Fig. 1A

![Fig. 1: Antioxidant activities of SOPs, DPPH scavenging activity (A), Fe²⁺ chelating capacity (B), ORAC (C), inhibiting the autoxidation of linoleic acid capacity (D). The results are expressed as mean ± SD of three experiments.](image)
with increasing peptide concentration. The ORAC value of SOPs was 446.1 μmol Trolox equivalents per gram dried weight (Fig. 1C). As shown in Fig. 1D, SOPs showed lipid peroxidation-inhibitory activity in a linoleic acid model system. Lower absorbance at 500 nm indicated higher lipid peroxidation inhibition. Overall, the results suggest SOPs exhibited potent antioxidant activity.

3.2. Protective effect of SOPs against H$_2$O$_2$ induced cytotoxicity

As the major ROS, H$_2$O$_2$ has been extensively used to induce oxidative stress, resulting in apoptosis or necrosis of PC12 cells. In our preliminary experiments, different concentrations of H$_2$O$_2$ (25 - 800 μM) induced injury on PC12 cells was assessed by MTS assay. As shown in Fig. 2A, the H$_2$O$_2$ treatment decreased the cell viability in a dose-dependent manner with 31.5 % viability at 200 μM H$_2$O$_2$ challenge which was used for further experiments. Then the neuroprotective effects of SOPs were investigated. The cells pretreated with different concentrations of SOPs (0.1, 0.5, 1 mg/mL) for 24 h before 200 μM H$_2$O$_2$ treatment (12 h) showed significant improvement in cell survival up to 49.2 ± 3.1% with 1 mg/mL of SOPs, as shown in Fig. 2B. These data shows that SOPs could increase cell viability and offer the protection against H$_2$O$_2$-induced cell death.

3.3. Protective effect of SOPs against plasma membrane damage

The cytotoxicity of H$_2$O$_2$ and the protective activity of SOPs were further evaluated by LDH assay. PC12 cells were pretreated with 1 mg/mL of SOPs for 24 h, before treatment with 200 μM H$_2$O$_2$ for 12 h (Fig. 2C). The results show that the release of LDH of 71.4 % of total enzyme with 200 μM H$_2$O$_2$ challenge which
indicates that H$_2$O$_2$ induces cytotoxicity in the PC12 cells. In contrast, SOPs pretreatment lowered the LDH release up to 29.4 % as compared with 200 μM H$_2$O$_2$-treated cells. The observed results demonstrate the protective effect of SOPs against 200 μM H$_2$O$_2$-induced neurotoxicity. The protective effect of SOPs was further more confirmed morphologically by bright field microscope. The 200 μM H$_2$O$_2$-challenged neurons exhibited cell shrinkage and disappearance of the cellular processes which was partially protected with pretreatment of SOPs (Fig. 2D).

4. Discussion

In the present study we observed the antioxidant and neuroprotective effects of SOPs. SOPs showed stronger inhibition of the autoxidation of linoleic acid and higher scavenging activity against 2,2-diphenyl-1-pircrylhydrazyl, superoxide free radicals, and highly capable of inhibiting H$_2$O$_2$-induced oxidative damage in PC12 cells. However, further work is being conducted to isolate and purify the bioactive peptides from SOPs, and clarify their structure-function relationship in order to elucidate the specific antioxidant pathway for cell protection.

5. Acknowledgements

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6. References


