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Antioxidant activity and protective effects of Alcalase-hydrolyzed soybean hydrolysate in human intestinal epithelial Caco-2 cells

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ABSTRACT

Soybeans are known as a promising source of bioactive peptides. However, knowledge on the antioxidant behaviors of soybean protein hydrolysate (SPH) in the human intestinal epithelium is limited. In this study, SPH was prepared with Alcalase and subsequently ultrafiltered into four peptide fractions as SPH-I (< 3 kDa), SPH-II (3–5 kDa), SPH-III (5–10 kDa) and SPH-IV (> 10 kDa). The antioxidant properties of SPH and membrane fractions were investigated using different chemical assays and their protective effects against oxidative stress were evaluated using H_2O_2-stressed human intestinal Caco-2 cells. Results showed that SPH-I exhibited the strongest 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical scavenging activity (IC_{50} = 2.56 mg/mL) and reducing capacity while SPH-III had the best metal ion-chelating activity (IC_{50} = 0.29 mg/mL). Both SPH and the peptide fractions dose-dependently suppressed intracellular reactive oxygen species (ROS) accumulation induced by H_2O_2 in Caco-2 cells, but the strongest inhibitory effect was observed for SPH-I. Amino acid (AA) results revealed that SPH-I was rich in hydrophobic and antioxidant AAs, which could contribute to its stronger antioxidant properties. Additionally, SPH-I protected Caco-2 cells from H_2O_2-induced oxidative stress *via* inhibiting lipid peroxidation and stimulating antioxidant enzyme activities. These results suggest that SPH-I and constitutive peptides can be beneficial ingredients with antioxidant properties and protective effects against ROS-mediated intestinal injury.

1. Introduction

It is well known that oxidative stress occurs when the accumulation of reactive oxygen species (ROS) beyonds the defense capacity of living cells, or when the cellular antioxidant system is insufficient (Hancock, Desikan, & Neill, 2001; Valko, Rhodes, Moncol, Izakovic, & Mazur, 2006). Excessive ROS can induce oxidative damage in cell membranes and biological molecules, which consequently leads to various chronic diseases, such as cardiovascular disease, diabetes, Alzheimer's and cancer (Valko et al., 2006; Waris & Ahsan, 2006). Dietary consumption of natural antioxidants has been shown as an effective way to attenuate the deleterious effects of ROS and restore the body's antioxidant load (Seifried, Anderson, Fisher, & Milner, 2007). Hence, significant interest has been spawned to generate food-derived protein hydrolysates with antioxidant properties. A number of protein hydrolysates and peptides have been investigated, including those from milk (Wang, Xie, & Li, 2016), egg white (Liu et al., 2014), cereal (Guo, Zhang, Jiang, Miao, & Mu, 2014) and fish (Je, Park, Hwang, & Ahn, 2015).

Soybean (*Glycine* max L.) is known as an important field crop in the world, providing quality protein with high nutritional value, desirable functional properties and low cost. Moreover, specific soy protein hydrolysates and peptides have been shown to have various antioxidant properties, such as free radical scavenging activity, metal ion-chelating activity and inhibitory effects on lipid peroxidation (Moure, Domínguez, & Parajó, 2006; Peñta-Ramos & Xiong, 2002; Zhang, Li, & Zhou, 2010). In addition, lunasin, a peptide derived from soybean, was reported to protect human intestinal Caco-2 cells against oxidative stress induced by hydrogen peroxide (H_2O_2) and *tert*-butylhydroperoxide (García-Nebot, Recio, & Hernández-Ledesma, 2014).

As the primary digestive system, the human intestine is a key source of ROS due to long-term exposure to environmental pollutants, toxins, food-derived oxidants and mutagens (Cross, Halliwell, & Allen, 1984). A large literature has established that the increased levels of ROS in intestinal cells are an essential pathology of various intestinal disorders, such as inflammatory bowel disease and colonic malignancies (Bhattacharyya, Chattopadhyay, Mitra, & Crowe, 2014). Therefore,

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considerable attention has been paid recently to evaluate the antioxidant effects of natural compounds towards the intestinal epithelium (Wang et al., 2016; Zhang, Liu, & Tsao, 2016). The human colon adenocarcinoma Caco-2 cell line is a reliable model for the human colonocytes and H_2O_2-stressed Caco-2 cells can reflect the physiological response of intestinal epithelium to food-derived antioxidants (Shi, Kovacs-Nolan, Jiang, Rong, & Mine, 2014; Shi, Kovacs-Nolan, Jiang, Tsao, & Mine, 2014; Wijeratne, And, & Schlegel, 2006). In a recent study, we portrayed an Alcalase-hydrolyzed soybean protein hydrolysate (SPH) with potential bioavailability and ROS-quenching ability in human Caco-2 cells (Zhang et al., 2018). Therefore, it is hypothesized that SPH and constitutive peptides may exert antioxidant and cytoprotective effects in the human intestinal epithelium. Bearing this in mind, the aims of the present study were to investigate the *in vitro* antioxidant activity of SPH, using both chemical methods and H_2O_2-stressed human intestinal Caco-2 cells. The impact of peptide size on measured antioxidant properties was also evaluated. In addition, the cytoprotective mechanism of the most active peptide fraction was explored and the potentially active peptides were identified.

2. Materials and methods

2.1. Materials

Full-fat soybean flakes were kindly provided by the Lanshan Group Corporation (Liaocheng, China). Alcalase 2.4 L (EC 3.4.21.62, 2.4 AU/g) was obtained from Novozymes (Beijing, China). 2,2-diphenyl-1-picrylhydrazyl (DPPH), ferrozine and 2′,7′-dichlorofluorescin diacetate (DCFH-DA) were purchased from Sigma–Aldrich (St. Louis, MO, USA). H_2O_2 was a product of Amresco (Solon, OH, USA).

The human colon adenocarcinoma-derived Caco-2 cell line was obtained from the American Type Culture Collection (ATCC). Dulbecco's modified Eagle medium (DMEM) with high glucose, non-essential amino acids (NEAA), penicillin/streptomycin, trypsin–EDTA and Hanks' Balanced Salt Solution (HBSS) were all products from Gibco Life Technologies (Grand Island, NY, USA). Fetal bovine serum (FBS) was bought from Pan Biotech (Aidenbach, Germany). A Cell Counting Kit-8 (CCK-8) was purchased from Nanjing Jiancheng Bioengineering Institute (Nanjing, China). Malondialdehyde (MDA), catalase (CAT), cellular glutathione peroxidase (GP) and glutathione reductase (GR) assay kits were purchased from Beyotime Institute of Biotechnology (Shanghai, China). All other chemicals and reagents used were of analytical grade.

2.2. Preparation of soybean protein hydrolysate (SPH) and membrane fractions

Preparation of SPH was conducted according to the method described in our previous work (Zhang et al., 2018). In brief, hydrolysis of soybeans was performed using Alcalase 2.4 L (1.85%, v/w) at pH 8.5, 55 °C for 3 h. Following reaction, the enzyme was inactivated by heating the mixture in boiling water for 10 min. After centrifugation, the aqueous fraction was collected, lyophilized, defatted and stored at −20 °C as SPH.

Ultrafiltration of SPH was conducted using disk membranes with molecular weight (MW) cut-offs (MWCO) of 3, 5, and 10 kDa (Millipore, Billerica, MA, USA). Four SPH fractions categorized as SPH-I (MW < 3 kDa), SPH-II (3–5 kDa), SPH-III (5–10 kDa) and SPH-IV (MW > 10 kDa) were collected, lyophilized and stored at −20 °C until further use. The peptide content was determined using the o-phthaldialdehyde (OPA) assay described by Zhu et al. (2013).

2.3. Amino acid (AA) composition

To analyze AA composition of SPH and membrane fractions, samples were hydrolyzed with 6 M HCl under nitrogen atmosphere at 110 °C for 24 h. After vacuum evaporation to remove the acid, the hydrolysates were appropriately diluted, filtered and analyzed with an automatic AA analyzer (Hitachi L-8800, Tokyo, Japan) according to Elfalleh et al. (2012). For sulfur-containing AAs (Met and Cys) determination, the samples were oxidized with performic acid prior to hydrolysis in HCl. Tryptophan was degraded and thus its content could not be detected using the aforementioned method. Final results of the AA composition were expressed as g/100 g protein.

2.4. Antioxidant activity

2.4.1. DPPH radical scavenging activity

The scavenging activity against DPPH radical was determined according to the method of Brand-Williams, Cuvelier, and Berset (1995), with some modifications according to Alashi et al. (2014). Briefly, samples were dissolved in 0.1 M sodium phosphate buffer (pH 7.0) containing 1% (w/v) Triton X-100 to obtain various peptide concentrations (0.5, 1.0, 2.0 and 5.0 mg/mL). 100 μL of DPPH solution (0.1 M in 95% methanol) was then mixed with 100 μL of sample solution in the 96-well microplates. After incubation at room temperature for 30 min in the dark, the absorbance was measured at 517 nm using a Tecan Infinite M200 microplate reader (Tecan Inc., Maennedorf, Switzerland). Buffer without added sample was used for the blank. The DPPH radical scavenging activity was expressed using the following equation:

$$\text{DPPH radical scavenging activity (\%)} = \left(\frac{A_b - A_s}{A_b}\right) \times 100 \quad (1)$$

where A_b and A_s represent absorbance of the blank and sample, respectively.

2.4.2. Metal ion-chelating activity

The metal ion-chelating activity was measured following the method of Xie, Huang, Xu, and Jin (2008), with minor modifications. Samples were prepared in distilled (DI) water to obtain various peptide concentrations (0.2, 0.3, 0.5 and 1.0 mg/mL). 1 mL of sample solution was premixed with 0.05 mL of 2 mM $FeCl_2$ and 1.85 mL of DI water in a reaction tube. Ferrozine solution (0.1 mL of 5 mM) was then added and mixed vigorously. The mixture was allowed to stand at room temperature for 10 min, after which the absorbance was detected spectrophotometrically at 562 nm. DI water without added sample was used for the blank. The percentage chelating effect was expressed using the following equation:

$$\text{Metal ion chelating activity (\%)} = \left(\frac{A_b - A_s}{A_b}\right) \times 100 \quad (2)$$

where A_b and A_s represent absorbance of the blank and sample, respectively.

2.4.3. Reducing power

The reducing power was evaluated using a previous method described by Arise et al. (2016). In brief, samples (2 mL) dissolved in 0.2 M sodium phosphate buffer (pH 6.6) were combined with 2 mL of 1% (w/v) potassium ferricyanide. The final peptide concentration in the assay was 0.5, 1.0, 2.0 and 5.0 mg/mL, respectively. Each resulting mixture was incubated at 50 °C for 20 min, followed by the addition of 2 mL of 10% (w/v) trichloroacetic acid. After centrifugation at 3000 *g* for 10 min, 2.0 mL supernatant was collected and mixed with 2.0 mL of DI water and 0.4 mL of 0.1% (w/v) $FeCl_3$. After 10 min, the absorbance was determined spectrophotometrically at 700 nm. Higher absorbance indicates higher reducing power of tested sample.

2.5. Cell culture and treatment

The Caco-2 cell line was cultured in DMEM supplemented with 10%