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Antioxidant and free radical-scavenging activities of wheat germ protein hydrolysates (WGPH) prepared with alcalase

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Abstract

Wheat germ protein hydrolysates (WGPH) were obtained by enzymatic hydrolysis of defatted wheat germ protein isolates using Alcalase 2.4L FG. The degree of hydrolysis (DH) of WGPH was determined to be about 25% using pH-stat method. The molecular mass distribution of WGPH was lower than 1500 Da. The antioxidant and free radical-scavenging activities of WGPH were investigated by employing several in vitro assay systems, including the linoleic acid emulsion model system, 1,1-diphenyl-2-picrylhydrazyl (DPPH)/superoxide/hydroxyl radical-scavenging, reducing power, and ferrous ion-chelating activity. The antioxidant activity of WGPH was close to that of α-tocopherol in a linoleic acid emulsion system. WGPH showed scavenging activity against free radicals such as DPPH, superoxide, and hydroxyl radicals. The radical-scavenging effect was in a dose-dependent manner, and the EC_{50} values for DPPH, superoxide, and hydroxyl radicals were found to be 1.30, 0.40 and 0.12 mg/mL, respectively. Moreover, WGPH also exhibited notable reducing power and strong chelating effect on Fe^{2+}. The data obtained by the in vitro systems obviously established the antioxidant potency of WGPH.

Keywords: Wheat germ protein hydrolysates; Antioxidant activity; Free radical-scavenging activity; DPPH; Alcalase

1. Introduction

Raw wheat germ, containing as much as 10% oil, is mainly used in food, medical and cosmetic industries as a source of oil [1]. The main by-product of the oil extraction process is a defatted wheat germ meal, which has relatively high protein content (∼30%) [2], making the defatted wheat germ meal as one of the most attractive and promising sources of vegetable proteins.

Annually, there is a rich production of wheat germ in the world. However, the precious wheat germ source has a poor utility for human applications, while the majority is used for animal feeding purposes. Thus, it is for this reason that efforts are being made to devise efficient methods for the recovery of proteins from defatted wheat germ meal and to prepare acceptable products for human consumption. One of the possible ways to utilize wheat germ proteins is to produce protein isolates by alkaline extraction and subsequent isoelectric precipitation—a procedure similar to soy protein isolates extraction [2]. However, wheat germ protein isolates have lower functional properties compared to soy and casein protein products [3]. Enzymatic hydrolysis is widely applied to improve and upgrade the functional and nutritional properties of food proteins. Claver and Zhou [4] reported that the recovery yield and functional properties of defatted wheat germ protein can be improved by various food grade proteases treatment, such as Alcalase, Flavourzyme, Papain, Neutrase, and Protamex. Their results also showed that Alcalase 2.4L was the most effective of the five proteases used. Pepsin and trypsin were reported to be able to improve the emulsifying activity and emulsifying stability of wheat germ protein [5].

In addition, various physiological activities have been detected in the hydrolysates derived from the proteolytic hydrolysis of many food proteins, such as antimicrobial, immunomodulatory, antihypertensive, antioxidant, opioide and mineral binding [6]. Several angiotensin I-converting enzyme inhibitory peptides from wheat germ hydrolyzate were reported by Matsui et al. [7]. In recent years, there is a growing interest to identify antioxidative properties in many natural sources

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including some dietary protein compounds due to the potential health hazards of some synthetic antioxidants. Up to now, numerous peptides derived from hydrolyzed food proteins have been shown to have noteworthy antioxidative activities against the peroxidation of lipids or fatty acids [8]. However, little information about the antioxidant and free radical-scavenging activities of wheat germ protein hydrolysates was available until now.

The present study reports on the antioxidant and free radical-scavenging activities of wheat germ protein hydrolysates (WGPH) prepared with Alcalase 2.4L FG. Different measurements, including the ability to inhibit the autoxidation of linoleic acid, the scavenging effect on free radical, reducing power, and ferrous ion-chelating activity are used to evaluate the antioxidant activities.

2. Materials and methods

2.1. Materials and chemicals

Raw wheat germ (RWG) was donated by Huaian Xinfeng Flour Mill (Jiangsu, China). Linoleic acid, α,α-diphenyl-β-picrylhydrazyl (DPPH), 2-deoxy-D-ribose, nitroblue tetrazolium salt, xanthine, xanthine oxidase (from buttermilk, 0.049 U/mL), α-tocopherol, ferrozine, butylated hydroxytoluene (BHT), and ascorbic acid were purchased from Sigma Chemical Co. (St. Louis, USA). Alcalase 2.4L FG was acquired from Novo Co. (Novo Nordisk, Bagsvaerd, Denmark). All other chemicals used in the experiments were of analytical grade.

2.2. Defatted wheat germ flours (DWGF) preparation

Raw wheat germ was selected and cleaned to remove contaminants. The enzymes of wheat germ were inactivated by heating for 20 min at 105 °C and it was then defatted with *n*-hexane for 8 h and air-dried at room temperature. The defatted wheat germ meal was milled using a laboratory scale hammer mill. The resulting flour (DWGF) was sieved through a 60-mesh screen and was kept in sealed glass jars at 4 °C until used.

2.3. Protein isolates preparation

Defatted wheat germ protein isolates were prepared according to the process described by Hettiarachchy et al. [3] with minor modifications. DWGF was dispersed in 1.0 mol/L NaCl solution (1:8, w/v) and stirred for 30 min at ambient temperature, and then its pH was adjusted to 9.5 by using 1 mol/L NaOH. After stirring for 30 min, the suspension was centrifuged at 8000 rpm for 20 min at 4 °C. The supernatant was adjusted to pH 4.0 with 1.0 mol/L HCl to precipitate the proteins, and centrifuged again at 8000 rpm for 20 min at 4 °C. The precipitates were washed several times with distilled water (pH 4.0), dispersed in a small amount of distilled water, and adjusted to pH 7.0 by using 0.1 mol/L NaOH. The dispersed product was freeze-dried.

2.4. Preparation of WGPH

The 10% (w/v) protein isolates solution was prepared and hydrolyzed with Alcalase for 6 h. The hydrolysis was carried out using the following hydrolysis parameters: enzyme–substrate ratio (E/S) = 0.4 AU/g of protein; temperature (T) = 50 °C; pH 8.0. The hydrolysis was conducted in a 200 mL reaction vessel, equipped with a stirrer, thermometer, and pH electrode. Hydrolysis was stopped by heat treatment at 90 °C for 10 min. Hydrolysates were clarified by centrifuging at 3000 rpm for 20 min to remove insoluble substrate fragments and residual enzyme. The hydrolysates were then frozen, lyophilized and stored at −20 °C before further analysis.

2.5. Degree of hydrolysis

The degree of hydrolysis, defined as the percentage of number of peptide bonds cleaved divided by the total number of peptide bonds in a protein, was calculated from the consumption of base (NaOH) by the pH-stat method of Adler-Nissen [9]. The percent DH was calculated by the following equation:

$$\mathrm{DH}(\%) = \frac{h}{h_{\mathrm{tot}}} \times 100 = \frac{\mathrm{BN_b}}{\alpha M_{\mathrm{p}} h_{\mathrm{tot}}} \times 100$$

where B and N_b represent the amount of NaOH consumed during the proteolysis of the substrate and its normality, respectively, α the average degree of dissociation of the α-NH_2 groups in the protein substrate (0.874 for 50 °C and pH 8.0), M_p the mass (g) of the protein (N × 5.7), and h_{tot} is the total number of peptide bonds available for proteolytic hydrolysis (7.8 m equiv./g).

2.6. Molecular weight distribution profile

Molecular weight distributions of WGPH were determined by gel permeation chromatography (GPC) using a high-performance liquid chromatography (HPLC) system (Waters 600, USA). A TSK gel2000 SW_{XL} column (7.8 i.d. × 300 mm, Tosoh, Tokyo, Japan) was equilibrated with 45% acetonitrile (v/v) in the presence of 0.1% trifluoroacetic acid. The hydrolysates (100 μg/50 μL) were applied to the column and eluted at a flow rate of 0.5 mL/min and monitored at 220 nm at room temperature. A molecular weight calibration curve was prepared from the average retention time of the following standards: cytochrome C (12,500 Da), aprotinin (6500 Da), bacitracin (1450 Da), tetrapeptide GGYR (451 Da), and tripeptide GGG (189 Da) (Sigma Co., St. Louis, MO, USA).

2.7. Inhibition of linoleic acid autoxidation

The antioxidative activity of WGPH with different periods of incubation was measured in a linoleic acid model system according to the methods of Osawa and Namiki [10] with some modifications. Each sample (10 mg) was dissolved in 10 mL 50 mM phosphate buffer (pH 7.0), and added to a solution of 0.15 mL linoleic acid and 10 mL 99.5% ethanol. Then, the total volume was adjusted to 25 mL with distilled water. The mixture was incubated in a conical flask with a screw cap at 40 ± 1 °C in a dark room, and the degree of oxidation was evaluated by measuring the ferric thiocyanate values. The ferric thiocyanate value was measured according to the method of Mitsuda et al. [11]. The reaction solution (100 mL) incubated in the linoleic acid model system described herein was mixed with 4.7 mL 75% ethanol, 0.1 mL 30% ammonium thiocyanate, and 0.1 mL 0.02 M ferrous chloride solution in 3.5% HCl. After 3 min, the thiocyanate value was measured by reading the absorbance at 500 nm following colour development with $FeCl_2$ and thiocyanate at different intervals during the incubation period at 40 ± 1 °C.

2.8. Amino acid analysis

For the determination of the amino acids, samples of WGPH and protein isolates (150 mg) were subjected to acid hydrolysis with 5 mL of 6 mol/L HCl under nitrogen atmosphere for 24 h at 110 °C. Each hydrolyzate was washed into a 50 mL volumetric flask and made up to the mark with distilled water. The amino acids were subjected to RP-HPLC analysis (Agilent1100, USA) after precolumn derivatization with *o*-phthaldialdehyde (OPA) [12] or with 9-fluorenylmethyl chloroformate (FMOC) [13].

Methionine and cysteine were determined separately as their oxidation products according to the performic acid procedure of Moore [14] prior to hydrolysis in 6N HCl. Amino acid composition was reported as g amino acid per 100 g protein.

2.9. Scavenging effect on DPPH free radical

The scavenging effect of WGPH on α,α-diphenyl-β-picrylhydrazyl (DPPH) free radical was measured according to the method of Shimada et al. [15] with some modifications. A volume of 2 mL of each sample was added to 2 mL of 0.1 mM DPPH in 95% ethanol. The mixture was shaken and left for 60 min at