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Characterization and in vitro antioxidation of papain hydrolysate from black-bone silky fowl (*Gallus gallus domesticus Brisson*) muscle and its fractions

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ABSTRACT

Black-bone silky fowl (*Gallus gallus domesticus Brisson*) (BSF) muscle was hydrolyzed by papain, and the hydrolysate was separated by preparative high performance liquid chromatography (HPLC). The amino acid composition of the BSF hydrolysate (BSFH) and its fractions was determined by HPLC precolumn derivation with 2,4-dinitrofluorobenzene. The molecular weight (MW) distribution of the BSFH and its fractions was measured by a peptide column on an HPLC system. Antioxidant activities of the BSFH and its fractions were studied by testing the reducing power and four radical scavenging systems: superoxide anion ($O_2^{\bullet -}$), hydroxyl (·OH), 1,1-diphenyl-2-picrylhydrazyl (DPPH•) and 2,2'-azinobis(3-ethylbenzothiazoline-6-sulfonic acid) ($ABTS^{\bullet +}$) radicals. The results demonstrated that the BSFH had strong antioxidant capacity to scavenge $O_2^{\bullet -}$, DPPH• and $ABTS^{\bullet +}$, and displayed strong reducing power, but revealed less powerful ability to scavenge ·OH. Fraction II of the BSFH exhibited the highest activity in scavenging $O_2^{\bullet -}$ and DPPH•, and reducing power, whereas fraction I displayed the strongest ·OH scavenging ability. Besides Glu, Asp and Gly, the rich amino acids of Ala and Leu played an important role in antioxidant activity. The small-size peptides with MW ranging from approximately 200–6000 Da probably contributed to higher antioxidant activity. Results from this study indicated that BSFH and its fractions could be used as food additives and diet nutrients.

1. Introduction

Free radical-mediated oxidation and antioxidants are receiving more and more attention in many current research areas. In the ground state, the oxygen molecule is stable until exposed to environmental pollutants, radiation, UV, etc. At this time, reactive oxygen species (ROS) and free radicals are easily formed, which can induce oxidative damage to biomacromolecules, including DNA, proteins, membrane lipids and carbohydrates (Wiseman & Halliwell, 1996; Lai & Piette, 1977; Kellogg & Fridovich, 1975). ROS and free radicals are reported to be involved in the occurrence of numerous diseases, such as cancer, atherosclerosis, diabetes, neurodegenerative disorders and aging (Halliwell, 1991; Pryor, 1982).

In food industry, lipid or fatty acid oxidation has been receiving great concerns because it can result in quality deterioration (development of undesirable off-flavor, discoloration, nutrition loss, formation of toxins), reducing the shelf-life of food products. To retard lipid or fatty acid oxidation, various antioxidants with a strong antioxidant capacity have been developed. Synthetic antioxidants, such as butylated hydroxyanisole (BHA) and butylated hydroxytoluene (BHT), can be used to preserve food containing fats and oils. However, because of their potential health risks and negative consumer perception (Yu et al., 2002), natural antioxidants have become appealing alternatives and are in great demand in the food industry. For example, tocopherols, ascorbate, flavonoids, carotenoids and phenolic compounds from plants are the most commonly used natural antioxidants in the processed food (Pokorný, 1991).

In recent years, it has been recognized that dietary proteins provide a rich source of naturally occurring antioxidants. These proteins are of wide distribution in plants, animals and fungi, such as soybean protein (Chen, Muramoto, Yamauchi, Fujimoto, & Nokihara, 1998), maize zein (Zhu, Chen, Tang, & Xiong, 2008), potato protein (Wang & Xiong, 2005), whey protein (Hernández-Ledesma, Dávalos, Bartolomé, & Amigo, 2005), grass carp muscle protein (Ren et al., 2008) and fermented mushroom protein (Sun, He, & Xie, 2004). Protein hydrolysates and peptides exhibit strong antioxidant efficiency in both model and in situ systems, including radical scavenging, reducing, and metal ion chelating activity (Elias, Kellerby, & Decker, 2008).

Black-bone silky fowl (*Gallus gallus domesticus Brisson*) (BSF), black in skin, meat, bones, and white in feathers, is a unique breed of chicken in China. It has been well known for its health functions, such as treating diabetes and anemia, curing women's diseases like menoxenia and postpartum complications. With the expansion of BSF breeding, effective development and utilization of BSF are emergent. Carnosine is a natural antioxidative di-peptide, present in

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a remarkably high content within BSF (Tian et al., 2007). However, little has been known about different antioxidant properties of hydrolysates and peptides derived from BSF muscle.

Because of antioxidant peptides production ability (Wang, Zhao, Zhao, & Jiang, 2007), easy availability and low commercial price, papain was used to hydrolyze BSF muscle. The antioxidant activities assessment can be achieved by means of determining their radical scavenging activity and reducing power. In the present study, the antioxidant properties of papain hydrolysates from BSF and its fractions separated by preparative HPLC were investigated in four radical scavenging systems (O_2•$^-$, ·OH, DPPH• and ABTS•$^+$) and reducing power using carnosine and ascorbate as comparison. In addition, the MW distribution and amino acid composition of the BSFH and its fractions were studied.

2. Materials and methods

2.1. Materials and reagents

Black-bone silky fowl (75–90 days old, half male and half female) were provided by the Taihe Original Black-Bone Silky Fowl Hennery (Jiangxi, China). Papain (320,000 U/g) was purchased from Beijing Fangshan Enzyme Factory (Beijing, China). Cytochrome *c*, 1,1-diphenyl-2-picrylhydrazyl (DPPH) and 2,2′-azinobis(3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt (ABTS) were purchased from Sigma Chemical Co. (St. Louis, MO, USA). Reduced and oxidized glutathione and 17 amino acid standards (Asp, Glu, Ser, Arg, Gly, Thr, Pro, Ala, Val, Met, Cys, Ile, Leu, Phe, His, Lys, Tyr) were purchased from Shanghai Sangon Biotech Co., Ltd. (Shanghai, China). Acetonitrile was of chromatographic grade. Other reagents used in this study were of analytical grade.

2.2. Preparation of BSFH

The enzymatic hydrolysis method optimized by uniform design was used to prepare BSFH. Briefly, BSF muscle (1/6, w/v) was hydrolyzed with papain (3‰, w/v) at 55 °C and pH 7.0 for 1 h to achieve a strong antioxidant activity. The enzyme was inactivated by heating at 90 °C for 20 min. The resulting BSFH solution was centrifuged at 3000×*g* for 10 min. The lipids and melanin were removed by chloroform-methanol (2:1, v/v) extraction and centrifugation. The supernatant was subjected to vacuum drying at 60 °C, sealed in plastic bags, and stored in a desiccator until use.

2.3. Preparative HPLC separation

The separation of BSFH was performed on a Waters DeltaPrep 400 preparative chromatography system equipped with Waters Prep LC controller and Waters 2487 dual λ absorbance detector (Waters, Milford, MA, USA). The preparative HPLC was performed on a Bondapak C_{18} preparative column (300×30 mm I.D., 10 μm). For the simple and fast separation, ultrapure water was selected as the mobile phase. The BSFH concentration was 50 mg/mL. The hydrolysate solution was filtered through a 0.45-μm Millipore membrane before injection. The injection volume was 5 mL. The flow rate was 25 mL/min, and the detected wavelength was 254 nm. The preparative HPLC equipment was controlled by Waters Empower 2 chromatography data software. The BSFH fractions were manually collected, reduced to 10 mL under reduced pressure rotary evaporation at 50 °C and then, freeze-dried, sealed, and stored in a desiccator before use.

2.4. Superoxide radical (O_2•$^-$) scavenging activity

The assay of O_2•$^-$ scavenging activity of the BSFH and its fractions were determined according to the autoxidation of a pyrogallol method described by Marklund and Marklund (1974). Briefly, 1.0 mL of samples (0.1, 1, 10 and 50 mg/mL for the BSFH; 1 mg/mL for each fraction) was mixed with 1.8 mL of 50 mmol/L Tris–HCl buffer (pH 8.2). The mixture was incubated at 25 °C for 10 min, and then 0.1 mL of 10 mmol/L pyrogallol (dissolved in 10 mmol/L HCl) was added. The absorbance of the solution at 320 nm was measured up to 4 min. The oxidation rate of pyrogallol for samples was calculated as the change of the absorbance (ΔA_1). The autoxidation rate of pyrogallol for control was measured with 1.0 mL of ultrapure water (ΔA_0). For comparison, the O_2•$^-$ scavenging activity of carnosine (0.1, 1 and 10 mg/mL) and ascorbate (0.1 and 1 mg/mL) was also tested. The O_2•$^-$ scavenging activity was calculated as $[(\Delta A_0 - \Delta A_1)/\Delta A_0] \times 100\%$.

2.5. Hydroxyl radical (·OH) scavenging activity

The ·OH scavenging assay of the BSFH and its fractions were carried out using the method described by Li, Jiang, Zhang, Mu, and Liu (2008) with some modifications. The sample tubes were filled out with the following solutions accordingly: 1 mL of 0.4 mol/L phosphate buffer (pH 7.4), 1 mL of 2.5 mmol/L 1,10-phenanthroline, 1 mL of samples (0.1, 1, 10 and 50 mg/mL of the BSFH; 1 mg/mL for each fraction), 1 mL of 2.5 mmol/L $FeSO_4$ and 0.5 mL of H_2O_2 (1%). The mixture was incubated at 37 °C for 1 h, and the absorbance was measured at 536 nm. The ·OH scavenging assay of carnosine (0.1, 1 and 10 mg/mL) and ascorbate (0.1 and 1 mg/mL) were also tested for comparison. The ·OH scavenging activity was calculated as $[(A_2 - A_0)/(A_1 - A_0)] \times 100\%$, where A_2 is the absorbance of the sample; A_0 is the absorbance of the blank solution using ultrapure water (1 mL) instead of sample solution (1 mL); and A_1 is the absorbance of the control solution using ultrapure water (1.5 mL) instead of sample solution (1 mL) and H_2O_2 (0.5 mL).

2.6. DPPH radical (DPPH•) scavenging activity

The DPPH• scavenging activity of the BSFH and its fractions was measured by using the procedure described by Chen, Xie, Nie, Li, and Wang (2008). The 0.2-mmol/L solution of DPPH in 95% ethanol was prepared daily before UV measurements. 1 mL of samples (0.1, 1, 10 and 50 mg/mL of the BSFH; 1 mg/mL for each fraction) was thoroughly mixed with 2 mL of freshly prepared DPPH and 2 mL of 95% ethanol. The mixture was shaken vigorously and allowed to stand for 30 min in the dark, and then the absorbance was measured at 517 nm against a blank. The DPPH• scavenging activity of carnosine (0.1, 1 and 10 mg/mL) and ascorbate (0.1 and 1 mg/mL) were also tested for comparison. The DPPH• scavenging activity was calculated as $[1 - (A_2 - A_1)/A_0] \times 100\%$, where A_0 is the absorbance of DPPH solution without sample (2 mL DPPH + 3 mL 95% ethanol), A_2 is the absorbance of the sample mixed with DPPH solution (1 mL sample + 2 mL DPPH + 2 mL 95% ethanol) and A_1 is the absorbance of the sample without DPPH solution (1 mL of sample + 4 mL 95% ethanol).

2.7. ABTS radical cation (ABTS•$^+$) scavenging activity

The ABTS•$^+$ scavenging activity of the BSFH and its fractions was analyzed by the method described by Re et al. (1999). This method is based on ABTS•$^+$ production by reacting ABTS stock solution (7 mmol/L) with potassium persulfate (2.45 mmol/L, final concentration). The mixture was left in the dark at room temperature for 12 h before use. One milliliter of samples (0.1, 1, 10 and 50 mg/mL of the BSFH; 1 mg/mL for each fraction) was mixed with 4 mL of ABTS•$^+$ solution, and the absorbance was recorded at 734 nm after 6 min. Carnosine (0.1, 1 and 10 mg/mL) and ascorbate (0.1 and 1 mg/mL) were also tested for comparison. The ABTS•$^+$ scavenging activity was calculated as $[(A_0 - A_1)/A_0] \times 100\%$, where A_0 is the absorbance of the blank solution without sample and A_1 is the absorbance of the sample solution.