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Antioxidant activity and characterization of bioactive polypeptides from bovine hair

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ABSTRACT

The antioxidant activity and food protection effect of bioactive polypeptides from bovine hair were investigated. In various *in vitro* tests, polypeptides showed a strong reducing power and a remarkable antioxidant capability to scavenge free radicals (ABTS, superoxide and hydroxyl radicals). Moreover, polypeptides also exhibited a significant food protection effect to inhibit the oxidation of edible oil by controlling the peroxide value (POV). Furthermore, by using gel filtration chromatography (GFC), high performance size exclusion chromatography (HPSEC), amino acid analysis and atomic force microscopy (AFM), an antioxidant polypeptide (APB) was purified and characterized with a molecular weight of 18.7 KDa in the form of spherical lumps, which was composed of 17 kinds of amino acids and contained sulfydryl group. Our results suggested that the antioxidant polypeptide from bovine hair could be a new potential source for preparing natural antioxidant applied in oil or oil-rich food.

1. Introduction

Oil oxidation is an autocatalytic reaction, which easily occurs in oil or oil-rich food. With the production of offensive odors and flavors, it can lead to the decrease of nutritional quality and safety of food [1]. Hazardous compounds generated in oil oxidation, such as free radicals and reactive aldehydes, are harmful to human health and associated with many chronic diseases [2]. One effective method to inhibit oil oxidation is the addition of antioxidants, such as butylated hydroxytoluene (BHT), butylated hydroxyanisole (BHA) and tertbutylhydroquinone (TBHQ). However, safety of these synthetic antioxidants has been questioned due to their toxic and carcinogenic effects exerted in animals and humans [3]. Thus, there is an increasing interest in novel antioxidants which can be applied safely. Antioxidant peptides are a possible solution. Recent studies have focused on the potential application of bioactive peptides as antioxidants in oil-rich food, due to their significant antioxidant activity and excellent solubility. On the other hand, it also has been applied as a source of bioactive components for functional foods and nutraceuticals [4].

Bovine hair, mainly composed of keratin, is a superior resource of protein. It is a by-product of food industry, which is commonly reused as feed material in agriculture [5]. With excellent biocompatibility and tough structure, bovine hair has been used as a novel biomaterial in medicine recently [6]. In addition, high content of cystine and disulfide linkage in the structure indicated that bioactive polypeptides from bovine hair might possess antioxidant capacity to scavenge free radicals and inhibit oil-oxidation. Unfortunately, there is no information about their antioxidant activity and food protection effect.

In present study, the antioxidant activity of polypeptides from bovine hair was evaluated using various experimental systems. In addition, their food protection effect to inhibit oil oxidation was also investigated. Further, gel filtration chromatography (GFC), high performance size exclusion chromatography (HPSEC), and atomic force microscopy (AFM) were employed to purify and characterize the antioxidant polypeptide.

2. Materials and methods

2.1. Materials and chemicals

Bovine hairs were derived from the back of adult yellow cattle (*Bos taurus domesticus Gmelin*, male) in a meat factory of Chengdu, China. Then, they were washed, dried at 50 °C for 3 days and finally stored under vacuum.

Butyl hydroxy anisd (BHA), dihydronicotineamidadenine dinucleotide (NADH), phenazine methosulfate (PMS), 2,2′-azinobis-3-ethylbenzthiazoline-6-sulfonate (ABTS), nitro blue tetrazolium (NBT), ethylene diamine tetraacetic acidwere (EDTA), deoxyribose and ascorbic acid (Vc) were purchased from Sigma (St. Louis, MO, USA). The solvents for HPSEC were of chromatographic purity. All other reagents used were of analytical grade.

2.2. Preparation of polypeptides

Bovine hair (10 g) was suspended in 200 mL of 0.5% (*w*/*v*) NaOH solution, and then stirred in water bath at 45 °C for 24 h. The

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homogenate obtained was filtered and centrifuged under 10,000 rpm at 4 °C for 20 min. Being dialyzed (MWCO 5000, Sigma, MO, USA) against ultrapure water until the pH value was at 7.0, polypeptides were prepared by freeze-drying, and then stored at −20 °C for further use.

2.3. Antioxidant activity of polypeptides

2.3.1. ABTS radical scavenging assay

ABTS radical cation solution was prepared through reaction of ABTS (7 mM) with potassium persulfate (2.45 mM) at 23 °C for 16 h in dark. The solution was diluted with pure water to obtain an absorbance of 0.700 ± 0.005 at 734 nm, and the diluted solution (3.9 mL) was then mixed with polypeptides solution (0.1 mL). The mixture was allowed to react at 23 °C for 6 min and the absorbance at 734 nm was recorded by using Lambda 25 UV–visible spectrophotometer (Perkin Elmer, CA, USA) [7]. The scavenging activity was calculated as $(1 - A_{\text{sample } 734}/A_{\text{control } 734}) \times 100\%$.

2.3.2. Superoxide radical scavenging assay

NADH (557 μM), PMS (45 μM) and NBT solutions (108 μM) were prepared by using Tris–HCl (16 mM, pH 8.0) as solvent. Polypeptides solution (0.1 mL) was mixed with 1 mL NADH, 1 mL PMS and 1 mL NBT. The mixture was incubated at 25 °C for 5 min and the absorbance was measured at 560 nm [8]. The scavenging activity was calculated as $(1 - A_{\text{sample } 560}/A_{\text{control } 560}) \times 100\%$.

2.3.3. Hydroxyl radical scavenging assay

Deoxyribose (2.67 mM) and EDTA (0.13 mM) were dissolved in phosphate buffered saline (PBS, 0.2 M, pH 7.4). The PBS solution (0.6 mL) was mixed with 0.1 mL polypeptides solution, 0.2 mL ferrous ammonium sulfate solution (0.4 mM), 0.05 mL Vc solution (2.0 mM) and 0.05 mL H_2O_2 solution (20 mM). The mixed solution was incubated at 37 °C for 15 min, and then 1 mL thiobarbituaric acid solution (1%, *w/v*) and 1 mL trichloroacetic acid solution (2%, *w/v*) were added. The mixture was boiled for 15 min and cooled in ice, and its absorbance was measured at 532 nm [9]. The scavenging activity was calculated as $(1 - A_{\text{sample } 532}/A_{\text{control } 532}) \times 100\%$.

2.3.4. Reducing power assay

Polypeptides solution (1 mL) was mixed with 2.5 mL PBS solution (0.2 M, pH 6.6) and 2.5 mL potassium ferricyanide solution (1%, *w*/*v*). The mixture was incubated at 50 °C for 20 min, followed by the addition of trichloroacetic acid solution (2 mL, 10%, *w/v*) and centrifugation at 2000 rpm for 15 min. A 2.5 mL of the supernatant was mixed with 2.5 mL water and 0.5 mL ferric chloride solution (0.1%, *w*/*v*), and the absorbance was measured at 700 nm [10].

2.4. Protective effect of polypeptides in food

Peanut oil and lard, purchased from a local market (Chengdu, China) were employed for oil-oxidation inhibitory test. Peanut oil (or lard, 25 g) was added to brown bottles and incubated at 45 °C for 30 min. Polypeptides was added to the oil to reach final concentrations of 0.01%, 0.05% and 0.1% (*w/w*). Then, the bottles without stopper were stirred and incubated at 70 °C. Sample without polypeptides was used as negative control and BHA (0.05%, *w/w*) was used as positive control.

The peroxide value (POV) of sample was determined according to a previous report [11]. Sample (2 g) was mixed with 30 mL acetic acid-chloroform solution (acetic acid:chloroform = 3:2, *v/v*) and 1 mL saturated potassium iodide solution (140%, *w/v*), and was incubated at 25 °C for 3 min. Subsequently, 100 mL pure water and 1 mL starch indicator (1%, *w*/*v*) were added and the mixture was titrated with sodium thiosulfate solution (STS, 0.002 M) until blue violet color disappeared. Reaction system without sample was used as blank control. Analyses were carried out after regular intervals of 24 h. POV was calculated according to the equation: POV (meq/kg) = $N \times (V_1 - V_2) \times (1000/M)$, there, N was STS concentration (M), V_1 was the volume of STS consumed by sample (mL), V_2 was the volume of STS consumed by blank control (mL) and M is sample quality (g).

2.5. Purification of antioxidant polypeptide

Polypeptides (1 g) was applied to a Sephadex G50 column (1.6 × 30 cm) and eluted using a linear gradient of 0–1 M NaCl solution at a flow rate of 0.3 mL/min. Eluate was monitored at 280 nm using a Lambda 25 UV–visible spectrophotometer (Perkin Elmer, CA, USA). Subsequently, antioxidant activity of each fraction was determined by ABTS radical scavenging assay. Active polypeptide (peak-II) which showed strong activity was collected, lyophilized, and further fractionated on a Toyopearl HW-55S column (1.6 × 30 cm) by eluting with Tris–HCl buffer (20 mM, pH 7.4) to arrive at homogenous preparation. Active polypeptide (peak-b) obtained at this stage was designated as "APB". The protein content at various stages of purification was detected by the Bradford's method [12]. Ninhydrin test was done with method described by Starcher and sulfydryl group was estimated by Ellman's test [13,14].

2.6. Characterization of APB

2.6.1. Homogeneity and molecular weight determination

The homogeneity and molecular weight of APB were determined by high performance size exclusion chromatography (HPSEC) using an Agilent 1100 HPLC system (Agilent Technologies, Ltd., CA, USA) equipped with a size exclusion chromatography (SEC) column (TSK gel Super SW 2000, 4.6 × 300 mm i. d. with a particle size of 4 μm, Tosoh, Tokyo, Japan). APB (5 mg/mL; 20 μL) was injected into the column and eluted at 25 °C by PBS (0.2 M, pH 6.8) containing 0.1 M Na_2SO_4 at a flow rate of 0.35 mL/min with dual wavelength detector at 280 nm. The molecular weight of APB was estimated in comparison with a calibration curve prepared by using standards (vitamin B_{12}, cytochrome C, chymotrypsin, ovoalbumin, bovine albumin, bovine trypsin, thyroglobulin, Sigma, MO, USA).

2.6.2. UV spectroscopy and amino acid composition analysis

APB solution (0.2 mg/mL, dissolved in ultrapure water) was scanned on a Lambda 25 UV–visible spectrophotometer (Perkin Elmer, CA, USA) from 200 nm to 400 nm. Ultrapure water was used as solvent and control.

APB was hydrolyzed using 6 N HCl at 120 °C for 24 h, and the amino acid composition was determined by an A300 fully automatic Amino acid analyzer (Membrapure, Berlin, Germany).

2.6.3. Observation with atomic force microscopy (AFM)

APB solution (50 μg/mL) was dropped on the surface of a mica sample carrier, allowed to dry and then was imaged in air at room temperature. The AFM used in this study was a SPM-9600 scanning probe microscope (Shimadzu Co., Kyoto, Japan) and was operated in the tapping-mode. The resulting imaging force was estimated to be 3–4 nN and the resonant frequency was about 2 kHz.

2.7. Statistical analysis

The data of all experiments were recorded as means ± standard deviations and were analyzed with SPSS (version 12.0 for Windows, SPSS Inc., CO, USA).