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Antioxidant activity of peptide fractions from whey protein hydrolysates as measured by electron spin resonance

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ABSTRACT

Whey protein isolate (WPI) was hydrolysed for 0.5–8 h using Alcalase, and the 5-h hydrolysate, identified to possess the strongest reducing ability, was subjected to column fractionation and antioxidant activity assays. Sephadex G-10 gel filtration chromatography of the 5-h hydrolysate yielded four fractions (I, II, III and IV) that were composed of peptides of >40*k*, 2.8–40*k*, 0.1–2.8*k*, and <0.1*k*, respectively. Fraction III exhibited the strongest free radical scavenging effects, which was evidenced by the electron spin resonance (ESR) of 1,1-diphenyl-2-picrylhydrazyl radicals (DPPH·) and of trapped hydroxyl (·OH) and superoxide ($O_2^{\cdot-}$) radicals. The results indicated that antioxidant activity of whey protein hydrolysates depended on peptide molecular weight, with peptides of 0.1–2.8*k* possessing the strongest radical scavenging activity.

1. Introduction

Free radicals are known to be involved in the oxidation of lipids, and oxidative decomposition of unsaturated fatty acids is a primary cause of the development of rancidity and shelf-life reduction in many foods. Free radicals can also modify DNA, proteins, and small cellular molecules and are believed to play a significant role in the occurrence of diseases, such as cardiovascular diseases, diabetes mellitus, neurological disorders, and even the Alzheimer's disease (Moskovitz, Yim, & Choke, 2002; Stadtman, 2006).

Free radical scavengers are preventive antioxidants. Synthetic antioxidants, such as butylated hydroxyanisole (BHA), butylated hydroxytoluene (BHT) and propyl gallate (PG), can be used to curtail free radical formation in food products thereby retarding lipid oxidation. However, the use of synthetic antioxidants is under increasing scrutiny due to the potential health risks associated with such compounds. Therefore, search for natural antioxidants as alternatives to synthetic ones is of great interest among researchers. Antioxidant activity has been identified in several food protein hydrolysates, including those derived from soybean protein (Andrés, Herminia, & Juan, 2006), zein protein (Kong & Xiong, 2006), egg yolk (Senji & Yumi, 2006), porcine haemoglobin (Chang, Wu, & Chiang, 2007), fish protein (Thiansilakul, Benjakul, & Shahidi, 2007), and canola protein (Cumby, Zhong, Naczk, & Shahidi, 2008).

Enzyme-hydrolysed whey protein is widely used as a bioactive and nutritional ingredient in health and food products (Marshall, 2004). Previous studies have shown that whey protein hydrolysates contain a broad range of antioxidant activity in an iron-catalysed liposome oxidation system (Peña-Ramos & Xiong, 2001) or a copper-catalysed liposome emulsion (Colbert & Decker, 1991), depending on the proteases used. Whey hydrolysates applied to cooked meat pork patties could suppress lipid oxidation (Peña-Ramos & Xiong, 2003). Individually, both hydrolysed lactalbumin and lactoglobulin could act as antioxidants (Hernández-Ledesma, Dávalos, Bartolomé, & Amigo, 2005).

Electron spin resonance (ESR) with a proper radical trapper is a sensitive method to detect protein and peptide radicals. The trapping technique is based on the measurement of transitions of unpaired electrons in a magnetic field, which can detect and identify molecules that have unpaired electrons, such as free radicals. ESR offers a sensitive, direct and accurate tool to monitor reactive species generated at room temperature. The high sensitivity of ESR allows the detection of low-concentration radicals in peptide samples, such as WPI hydrolysate fractions. Park, Shahidi, and Jeon (2004) evaluated the activity of enzymatic extracts from an edible seaweed (*Sargassum horneri*) to scavenge DPPH, hydroxyl, and alkyl radicals by means of ESR spectroscopy. The results showed a strong efficacy of the extracts in quenching all radicals.

The aim of this study was twofold: first, to produce a potent antioxidative hydrolysate from WPI by treating the substrate with Alcalase for various time periods, and then, to elucidate the mode

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of action of the antioxidative hydrolysate by means of peptide fractionation followed by testing their scavenging capability against DPPH, hydroxyl and superoxide radicals with ESR.

2. Materials and methods

2.1. Materials

Whey protein isolate (WPI, 95% protein) was obtained from Davisco Foods International, Inc., USA. Alcalase 2.4 L (6×10^4 μ/g) was from Novozymes (Franklinton, NC). The testing chemicals, all reagent grade, including ferrous sulphate ($FeSO_4 \cdot 7H_2O$), BHA (2-tert-butyl-4-methoxyphenol), 1,1-diphenyl-2-pycryl hydrazyl (DPPH), 2,4,6-tris(2-pyridyl)-*s*-triazine (TPTZ), 5,5-dimethyl-1-pyrroline-*N*-oxide (DMPO, a spin trapper), disodium ethylenediaminetetraacetate (EDTA), and riboflavin, were purchased from Sigma Chemical Co. (St. Louis, MO). Other chemicals and reagents were also of analytical grade.

2.2. Enzymatic hydrolysis

To produce antioxidant peptides, WPI samples (45 mg/mL), with heat pretreatment (95 °C, 5 min), was hydrolysed using Alcalase for 0.5, 1, 2, 3, 5, and 8 h, at the optimal condition (pH 8.5, 65 °C). During hydrolysis, the pH of the broths was adjusted every 15 min to the optimal value with 1 N NaOH. The enzyme:WPI ratio (*E*/*S*) was 2:100. After hydrolysis, the pH of the broths was brought to 7.0, and the solutions were then heated in boiling water for 10 min to inactivate the enzyme. The hydrolysates were freeze dried (LGJ-1 Freeze-Dryer, Shanghai, China), and the lyophilised powders were stored at 4 °C before use.

Degree of hydrolysis (DH) of hydrolysed WPI was determined using a pH-stat method (Adler-Nissen, 1986) based on the equation: $DH = (h \div h_{tot}) \times 100\%$, where $h = B \times N_b \times 1/\alpha \times 1/MP$, B = base consumption (mL), N_b = concentration of base (1 M NaOH), $1/\alpha$ = calibration factors for pH-stat ($1/\alpha = 1.01$ for Alcalase), MP = mass of protein (g), and h = hydrolysis equivalents. For whey, h_{tot} = 8.8 mmol/g protein.

2.3. Reduction power

The reducing ability of hydrolysed whey was measured using the ferric reducing/antioxidant power (FRAP) assay (Benzie & Strain, 1996). Aliquots of 3.0 mL each of freshly prepared FRAP reagent were mixed with 0.3 mL of distilled water and 0.1 mL of hydrolysed WPI (45 mg/mL) solution reconstituted from the freeze dried powder. The FRAP reagent contained 2.5 mL of 10 mM TPTZ solution in 40 mM HCl plus 2.5 mL of 20 mM $FeCl_3 \cdot 6H_2O$ and 25 mL of 0.3 mM acetate buffer (pH 3.6). Absorbance (593 nm) of sample as well as blank solutions was taken at 30 s intervals for up to 8 min. Sample FRAP values were calculated based on a $FeSO_4$ standard curve prepared with 100–1000 μM $FeSO_4 \cdot 7H_2O$, and were expressed as $FeSO_4$ equivalent (μM).

2.4. Size exclusion chromatography

The molecular mass distribution of 5-h hydrolysate samples was separated on a Sephadex G-10 (2.6 × 65 cm) column by using protein purifier (BioLogic LP, BIO-RAD Laboratories, Inc., USA). Phosphate buffer (PBS, 10 mM, pH 7.4) solution was used to equilibrate the column and to elute the proteins at a flow rate of 0.5 mL/min. A fixed amount of sample (1.5 mL) at a protein concentration of 45 mg/mL was applied to the column, and 3-mL fractions were collected. The absorbance of the effluent was measured at 280 nm. Blue dextran (2*k*), vitamin B_{12} (1.355*k*), bovine serum albumin (66*k*), cytochrome C (12*k*), insulin A (2.5*k*), and tryptophan (0.204*k*) as molecular weight standards (Sigma Chemical Co., St. Louis, MO) were also chromatographed, separately. The eluents for each fraction were pooled and protein concentrations measured by the method of Lowry, Rosebrough, Farr, and Randall (1951). The mixtures, after being standardised to a 1 mg/mL protein concentration, were immediately subjected to RSA tests.

2.5. Determination of antioxidant activity by ESR spectroscopy

2.5.1. DPPH radical scavenging

The DPPH radical scavenging activity (RSA) of protein hydrolysates was measured using the method described by Nanjo et al. (1996). A 60 μL (1 mg/mL protein concentration) peptide solution (or ethanol itself as control) was added to 60 μL of DPPH (60 μM) in ethanol solution. After mixing vigorously for 10 s, the solution was transferred into a 100 μL quartz capillary tube, and the spin adduct was measured on an ER 200D-SRC ESR spectrometer (Bruker, Germany) exactly 2 min later. Ascorbic acid was used as standard positive control. Experimental conditions were as follows: magnetic field, 3385 Gs; power, 20 mW; modulation frequency, 100 kHz; amplitude, 1 Gs; and sweep time, 300 s. DPPH RSA was calculated based on the following equation, in which H and H_0 were the height of the third resonance peak for samples with and without protein, respectively.

$$RSA = (H_0 - H)/H_0 \times 100\%.$$

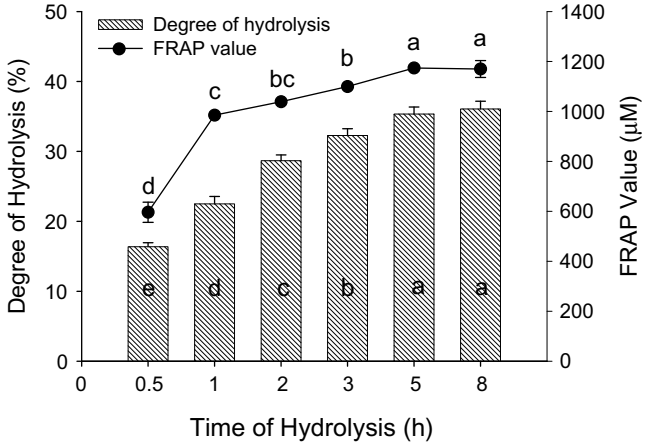

Fig. 1. Degree of hydrolysis of preheated WPI treated with Alcalase and it's reducing activity. Means with different letters (a–e) differ significantly ($P < 0.05$).

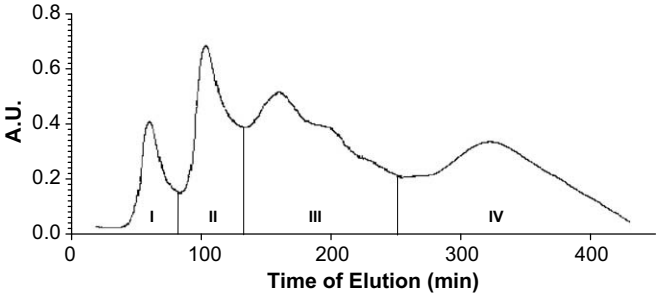

Fig. 2. Gel filtration pattern of WPI 5h-hydrolysate.