Biochemical and Biophysical Research Communications xxx (2014) xxx–xxx

Contents lists available at ScienceDirect

Biochemical and Biophysical Research Communications

journal homepage: www.elsevier.com/locate/ybbrc

Purification and characterization of high antioxidant peptides from duck egg white protein hydrolysates

Yao Ren [a,b], Hui Wu [b], Xiaofeng Li [a,*], Furao Lai [b], Xinglong Xiao [b,**]

[a] State Key Laboratory of Pulp and Paper Engineering, South China University of Technology, Guangzhou 510641, China
[b] College of Light Industry and Food Sciences, South China University of Technology, Guangzhou 510641, China

ARTICLE INFO

Article history:
Received 19 August 2014
Available online xxxx

Keywords:
Antioxidant activity
Chromatography
Duck egg white protein
Bioactive peptide
Isolation and purification

ABSTRACT

The hydrolysate from duck egg white protein (DEWP) prepared by "SEEP–Alcalase" at degree of hydrolysis (DH) value of 21% (namely HSA_{21}) exhibited high antioxidant capacity in different oxidation systems. A consecutive chromatographic method was then developed for separation and purification of HSA_{21}, including ion-exchange chromatography, macroporous adsorption resin (MAR) and gel filter chromatography. The final peptides "$P_{21\text{-}3\text{-}75\text{-}B}$" were obtained with significantly enhanced antioxidant activity ($p < 0.05$). It was further confirmed that the product mainly consisted of five oligopeptides (Mr: 202.1, 294.1, 382.1, 426.3, and 514.4 Da). Furthermore, the antioxidant activity of $P_{21\text{-}3\text{-}75\text{-}B}$ kept stable after *in vitro* digestive simulation. Antioxidant capacity of the purified peptides was closely related to the molecular mass, hydrophobic amino acid residues, acidic amino acid and some antioxidant amino acids. This research provided a valuable route for producing new natural-source peptides with strong antioxidant capacity and high nutritious value for our daily intake.

1. Introduction

Naturally, there is a dynamic balance between free radicals and antioxidant substances in human bodies [1]. Once the antioxidant enzymes, such as superoxide dismutase and catalase, cannot scavenge free radicals promptly, the balance may be disrupted, leading to cells or tissues injury, even cardiovascular diseases and cancer [2]. Thus, it is a present urgent requirement to search a safe, easily absorbed, nutrient and antioxidant edible.

Duck egg is one of the most commonly used eggs in food manufactures in China. A large amount of duck egg white protein (DEWP) was left as byproduct. Considering the quantity and proportion of essential amino acids, DEWP was an ideal edible protein suitable to be absorbed in human intestinal tract [3]. Therefore, it was chosen to prepare peptides with high antioxidant activity and additional value by enzymatic hydrolysis. Although the hydrolysates from DEWP displayed high antioxidant activity [4], abundant impurities and some low antioxidant peptides were included excepted for meritorious peptides. The application of separation and purification procedures was very essential to remove these undesirable components for improving the bioactivity and determining the composition of novel peptides expediently [5,6]. However, there is still little information regarding the isolation and purification of the DEWP hydrolysates.

In our ongoing research related to the preparation of natural-source peptides with high activities [4], we developed a consecutive chromatographic method for separation and purification of an antioxidant hydrolysate from DEWP by macroporous adsorption resin chromatography, ion-exchange chromatography and gel filer chromatography. The antioxidant activities of all the fractions from every purification process were evaluated and compared in different assays, including DPPH·, ·OH, O_2^{-}· radical scavenging activity and reducing power. Furthermore, the molecular mass and amino acids composition of the purified peptides were determined by electron spray ionization tandem mass spectrometry (ESI-MS) and amino acid analyzer.

2. Materials and methods

2.1. Materials and chemicals

Fresh duck egg was provided by Guangzhou Agricultural Institute, China. Alcalase 2.4 L FG (Alcalase) and Pancreatin was purchased from Novo Enzyme Co., Denmark, A hydrolase specific for egg protein (SEEP) was purchased from Waldorf Co., China. DPPH was purchased from Johnson Matthey Co., U.K. DEAE sepharose

* Corresponding author. Address: State Key Laboratory of Pulp and Paper Engineering, South China University of Technology, Wushan Road 381, Guangzhou 510641, China. Fax: +86 20 87111770.
E-mail addresses: xflibio@gmail.com, xflibio@scut.edu.cn (X. Li).

http://dx.doi.org/10.1016/j.bbrc.2014.08.116
0006-291X/© 2014 Published by Elsevier Inc.

FF was purchased from GE Co. America. Macro absorption resin (MAR) "DA201-C" was purchased from JiangYin Organic Chemical Plant, China. Sephadex G-15 was purchased from Sigma Co., America. All other chemicals and reagents were of analytical grade commercially available.

2.2. Preparation of DEWP hydrolysates

DEWP was isolated from fresh duck eggs, and then dissolved in distilled water with a final concentration of 50 mg/mL and pretreated at 95 °C for 40 min (pH 10). Two-step enzymatic procedure was applied, the reaction catalyzed by SEEP–Alcalase. The optimization of specific parameters has been discussed in previous paper [4]. And the final hydrolysates were centrifuged at 10,000*g* for 10 min using a high speed refrigerated centrifuge (Eppendorf Co., Germany). The supernatant was freeze-dried using a freeze dryer (Christ Co., Germany), collected and stored at −18 °C.

2.3. Determination of degree of hydrolysis

When SEEP or Alcalase was used as biocatalyst, the reaction pH value was >7.0 and DH value was measured with "pH-stat" method as follows:

$$\text{Degree of hydrolysis (DH, \%)} = B \times \text{Nb} \times \frac{1}{\alpha} \times \frac{1}{\text{MP}} \times \frac{1}{h_{\text{tot}}} \times 100\% \quad (1)$$

where B is the volume of NaOH solution consumed, mL; Nb is the concentration of NaOH solution consumed, mmol/mL; α is the degree of dissociation of α-amino acid, $\alpha = [10(\text{pH} - \text{pK})]/[1 + 10(\text{pH} - \text{pK})]$ (the average pK of the amino, 7.0; pH, the value of the initial reaction). MP is the total mass content of the substrate protein (g). The h_{tot} value is the total quantity of peptide linkage unit of substrate protein, mmol/g, calculated from amino acid analysis by summing the molar concentrations of each individual amino acid (mmoles) per gram of egg white protein [7].

2.4. DPPH· radical scavenging activity assay [8]

To test tube A, 2 mL ethanol and 2 mL DPPH·-ethanol solution (20 mM) were added and incubated in darkness at room temperature for 30 min. The absorbance of the resulting solution A was measured at 517 nm using a UV 752S spectrophotometer (Lengguang Co., Shanghai, China) with ethanol as control. 2 mL of the sample solution (1 mg/mL) was mixed with 2 mL of 20 mM DPPH·-ethanol solution, giving solution B which was also incubated for 30 min in darkness at room temperature and measured at 517 nm. Determination of VC was as a positive control. The IC_{50} value was used to express the DPPH· radical scavenging activity of samples, which was the concentration of tested samples required for a scavenging rate of 50%. DPPH· radical scavenging activities of the samples were calculated as follows:

$$\text{Scavenging activity (\%)} = \frac{A_0 - (A_x - A_{x0})}{A_0} \times 100\% \quad (2)$$

where A_0 is the absorbance of solution A; A_x is the absorbance of solution B; A_{x0} is the absorbance of 1 mg/mL sample solution.

2.5. ·OH radical scavenging activity assay

The reaction was initiated by adding 2 mL of 8.8 mM H_2O_2 to the reaction mixture containing 2 mL of 9 mM $FeSO_4$, 2 mL of 9 mM salicylic acid in ethanol and 2 mL of hydrolysate (1 mg/mL). The reaction mixture (solution A) and distilled water (the control, solution B) were incubated at 37 °C for 30 min in a water bath, and their absorbance were measured at 510 nm. Determination of VC was as a positive control. ·OH radical scavenging activities of the samples were calculated as follows:

$$\text{Scavenging activity (\%)} = \frac{A_0 - (A_x - A_{x0})}{A_0} \times 100\% \quad (3)$$

where A_0 is the absorbance of solution B, Ax is the absorbance of solution A, Ax_0 is the absorbance of 1 mg/mL sample solution.

2.6. Determination of O_2^{-}· radical scavenging activity

The mixture containing 0.2 mL of hydrolysate (1 mg/mL), 4.5 mL of 50 mM Tris–HCl buffer (pH 8.2) and 4 mL of distilled water, was incubated at 25 °C for 10 min and then mixed with 0.3 mL of 3 mM pyrogallic acid solution, forming solution A. When the hydrolyzed sample was replaced by distilled water, the resulted solution was named as solution B. Determination of VC was as a positive control. O_2^{-}· radical scavenging activities of the samples were calculated as follows:

$$\text{Scavenging activity (\%)} = \frac{K_0 - K_1}{K_0} \times 100\% \quad (4)$$

where K_0 is the slope of absorbance variation of solution B in 5 min, K_1 is the slope of absorbance variation of solution A.

2.7. Determination of reducing power on Fe^{3+}

The reducing power was measured in terms of the absorbance at 700 nm according to "Siddhuraju" method [9]. 2 mL of sample (1 mg/mL) and 1.0% (w/v) $K_4Fe(CN)_6$ were added into 2 mL of 0.05 M phosphate buffer solution (pH 6.6). The mixture was incubated at 50 °C for 20 min followed by the addition of 2.5 mL of 10% (w/v) TCA, centrifuged at 5000 g for 10 min. 2.5 mL of supernatant, 0.5 mL of 0.1% (w/v) $FeCl_3$ and 2.5 mL of distilled water were mixed for a 10 min reaction. Then the absorbance of resulting solution was measured at 700 nm. Determination of VC was as a positive control.

2.8. Ion-exchange chromatography

The hydrolysate (0.5 g) was dissolved in 10 mL of 10 mM Tris–HCl buffer (pH 7.6) and loaded onto an ion-exchange column (2.6 × 60 cm) previously packed with DEAE sepharose FF and equilibrated with the above-mentioned buffer at a flow rate of 48 mL/h continually with the effluent monitored at 214 nm. Then 0–1.0 M NaCl solution in 10 mM Tris–HCl butter (pH 7.6) were loaded onto the chromatography column (2.6 × 60 cm) with a linear gradient at a flow rate of 48 mL/h. 8 mL of each fraction was collected and monitored at 214 nm. The fraction (namely A) with the desired peak was freeze-dried and collected for the following purification.

2.9. Macroporous adsorption resin

Fraction A of 100 mg/mL was loaded at a flow rate of 60 mL/h onto the chromatography column (2.6 × 60 cm) packed with MAR "DA-201C" continually and the effluent monitored at 214 nm. The addition of the hydrolysates was stopped, when the absorption value reached higher than 0.1. Then the ethanol solution with the concentration of 25% (v/v) was loaded onto the chromatography column at the same flow rate of 50 mL/h. When the absorbance of the effluent at 214 nm exceeded 0.1, the eluting solvent (25% ethanol) was substitute for 50%, 75% and 100% (v/v) ethanol in sequence. The fraction (namely B) with the desired peak was freeze-dried, and collected for the further purification.

Please cite this article in press as: Y. Ren et al., Purification and characterization of high antioxidant peptides from duck egg white protein hydrolysates, Biochem. Biophys. Res. Commun. (2014), http://dx.doi.org/10.1016/j.bbrc.2014.08.116