Food Chemistry 188 (2015) 467–472

Contents lists available at ScienceDirect

Food Chemistry

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

Purification and characterization of antioxidant peptides from enzymatically hydrolyzed chicken egg white

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ARTICLE INFO

Article history:
Received 19 August 2014
Received in revised form 5 March 2015
Accepted 5 May 2015
Available online 6 May 2015

Keywords:
Chicken egg white
Enzymatic hydrolysis
Ultrafiltration
Chromatographic separation
Antioxidant peptides

ABSTRACT

Egg white is considered as a rich source of high quality proteins with various bioactive peptide fractions. Enzymatic hydrolysis of proteins can be used to release bioactive fractions and different enzymes have different abilities in releasing such bioactive fractions depending on the enzyme's site of activity on a protein. In this study, several proteases were examined for their ability to release antioxidant peptides from hen egg white and protease P was selected based on the antioxidant activity and the digestion yield of the crude protein hydrolysate. A combination of several purification steps including ultrafiltration with low molecular weight cut-off membranes, cation exchange chromatography and reversed phase high performance liquid chromatography was used to purify 'protease P egg white hydrolysate'. Sixteen antioxidant peptides, which were derived from ovalbumin, ovotransferrin and cystatin were isolated from the most active fractions. Amino acid sequences of those peptides were determined using *LC–MS/MS*. Oxygen radical absorbance capacity (ORAC) values of selected short chain peptides were determined using synthetic peptides. Two peptides AEERYP and DEDTQAMP (Ala-Glu-Glu-Arg-Tyr-Pro and Asp-Glu-Asp-Thr-Gln-Ala-Met-Pro) showed the highest ORAC values. The results from this study indicate that egg white is rich in antioxidant peptides which can be used as a potential source for preparing bioactive ingredients using enzymatic hydrolysis followed by purification techniques.

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1. Introduction

Food-derived bioactive compounds, such as bioactive peptides, omega-3 fatty acids, flavonoids, phenolic compounds etc., are a major research focus in the field of food science and nutritional science. These compounds exhibit antihypertensive, antioxidant, anti-inflammatory, antimicrobial, immuno-modulatory and other biologically relevant activities that might benefit human health and well-being (Liu, 2004; Pan, Lai, Dushenkov, & Ho, 2009; Sacks et al., 2006). Because oxidative stress is related to the onset and development of various chronic diseases, antioxidants have been extensively explored as a potential remedy to reduce the risk of chronic diseases. Although controlled physiologically production of free radicals is essential for many signaling pathways and normal functions, an imbalance between the formation of radicals and endogenous antioxidative system will induce oxidative damage to biomolecules, such as lipids, nucleic acids, proteins and carbohydrates, leading to cellular damage and diseases (Halliwell, 1994; Pacifici & Davies, 2009; Sarmadi & Ismail, 2010; Soobrattee, Neergheen, Luximon-Ramma, Aruoma, & Bahorun, 2005). In addition, antioxidants are believed to play a role in preventing or delaying autoxidation of food components and widely used in different food commodities (Shahidi & Zhong, 2010).

Phytochemicals such as phenolic compounds and flavonoids are well-known antioxidants. Antioxidant peptides, due to their nutritional value and possible functional properties such as emulsification and foaming, present additional advantages over phytochemical antioxidants (Xie, Huang, Xu, & Jin, 2008). Therefore, there is an increasing interest in developing antioxidant peptides. Bioactive peptide is usually inactive when exists as a part of the parent protein but can be released during food processing and photolytic hydrolysis. The size and composition of a peptide determine its antioxidant and free radical scavenging properties (Guo, Kouzuma, & Yonekura, 2009). Antioxidant peptides have been reported from soy protein (Chen, Muramoto, Yamauchi, & Nokihara, 1996; Moure, Dominguez, & Parajo, 2006), wheat protein (Zhu, Zhou, & Qian, 2006; Wang, Zhao, Zhao, & Jiang, 2007), fish protein (Bougatef et al., 2010; Je, Qian, Byun, & Kim, 2007; Kim, Je, & Kim, 2007), milk (Pihlanto, 2006), and egg white protein (Davalos, Miguel, Bartolomé, & López-Fandiño, 2004; Shen, Chahal, Majumder, You, & Wu, 2010; You & Wu, 2011).

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http://dx.doi.org/10.1016/j.foodchem.2015.05.014
0308-8146/© 2015 Published by Elsevier Ltd.

Chicken egg white is an excellent source of high quality proteins and is also a good source of bioactive peptides. Generally, one or several proteases are used to prepare proteins hydrolysate, which will then be subjected to a series of purification steps together with bioactivity assays to separate potent bioactive peptides. Antioxidant peptides were characterized from pepsin digested egg white hydrolysate (YAEERYPIL and SALAM) (Davalos et al., 2004), thermolytic hydrolysates of ovotransferrin (14 peptides, Shen et al., 2010), trypsin and papain hydrolysates of lysozyme (Memarpoor-Yazdi, Asoodeh, & Chamani, 2012) and ovalbumin (Xu, Shangguan, Wang, & Chen, 2007). To generate more potent antioxidant peptides, it is necessary to use the right protease to hydrolyze whole egg white protein. Our previous studies suggested that thermolysin and alcalase prepared egg white hydrolysates displayed lower antioxidant activity than those prepared by gastrointestinal proteases (You & Wu, 2011). Although gastrointestinal (GI) enzymes have been widely studied to produce egg bioactive peptides, the potential of egg white protein for preparing antioxidant peptides has not been fully explored, as it is unclear if the use of other proteases might generate more potent antioxidant peptides from egg proteins. Furthermore, many of the peptides have not been characterized. Therefore, the objectives of the study were to determine the appropriate protease for preparing egg white protein hydrolysate and further to purify and characterize the antioxidant peptides.

2. Materials and methods

2.1. Materials

Egg white powder, 2,2′-Azobis(2-methylpropionamidine) dihydrochloride (AAPH), Fluorescein and Trolox® were obtained from Acros Organics (Morris Plains, N.J., U.S.A.). Protease P "Amano" 6 (from *Aspergillus melleus*), protease M "Amano" (from *Aspergillus oryzae var.*), and protease S "Amano" G (from *Bacillus stearothermophilus*) were all kindly provided by Amano Pharmaceuticals Co. (Nagoya, Japan), Protex 51FP (from *A. oryzae var.*) was obtained from Genencor International, a Division of Danisco (Rochester, NY), alcalase (from *Bacillus licheniformis*) was purchased from Sigma–Aldrich ® (Oakville, ON, Canada). Enzyme activity and working conditions was listed in Table 1. Interesting peptides were chemically synthesized by GenScript (Piscataway, NJ) and their purity was determined to be greater than >95% by LC–MS.

2.2. Preparation of egg white protein hydrolysates

Egg white powder was added into distilled water to obtain 50 mg/mL (w/v) protein slurry. The slurry was heated at 90 °C for 10 min with continuous stirring to avoid gelation, and then the pH and the temperature was adjusted to the values recommended by the manufacturer for each enzyme (see Table 1). When the conditions were stabilized, the enzyme was added at a ratio of 25:1 (w/w, egg white powder to protease). All digestions were performed in a jacketed beaker connected to circulating water bath for maintaining constant temperature and Titrando® (Metrohm, Herisan, Switzerland) for maintaining constant pH with 0.5 M NaOH or 0.5 M HCl. After 3 h digestion, the hydrolysate was heated at 95 °C for 10 min to terminate the enzymatic activity. The hydrolysate was centrifuged at 10,000*g* for 25 min and the supernatants were freeze-dried and used for further analysis. Digestion yield was calculated based on the following equation (Dey & Dora, 2014).

$$\text{Digestion yield}\,(\%) = \frac{\text{weight of protein hydrolysate after centrifugation}}{\text{weight of protein subjected to digestion}} \times 100$$

2.3. Purification of antioxidant peptides from egg white hydrolysate

2.3.1. Ultrafiltration

The protease P egg white hydrolysate was fractionated through ultrafiltration, using the membrane with molecular weight cut off (MWCO) of 3000 Da (Millipore, USA). Permeate was freeze-dried to be used for further purification.

2.3.2. Cation exchange chromatography

The permeate (3 kDa) was dissolved in 0.01 M ammonium acetate buffer at a concentration of 100 mg/mL and filtered through 0.2 μm filter and subjected to cation exchange chromatography on a cation exchange column (16 × 100 mm, HiPrep 16/10 SP FF, GE Healthcare) operated by AKTA explorer 10XT system (GE Healthcare, Uppsala, Sweden). The column was equilibrated with 10 mM ammonium acetate (pH 4) over 5 column volumes (CV) and then 4 mL of sample was injected onto the column. After washing the column with 2 CVs of 10 mM ammonium acetate (pH 4), the sample was eluted with a linear gradient of 0–10% 0.5 M ammonium carbonate over 5 CV at a flow rate of 5 mL/min. The elution was monitored at 280 nm. Each peptide fraction was pooled, freeze dried, and subjected to activity test to identify the most active fraction.

2.3.3. RP-HPLC

The peptide fraction showed the highest antioxidant activity after cation exchange chromatography was further purified using reverse-phase high performance liquid chromatography (RP-HPLC) on an Xbridge C_{18} column (10 × 150 mm, 5 μm, Waters Inc., Milford, MA, USA) coupled with a guard column (10 × 40 mm) on Waters 600 system (Waters, Millford, MA) equipped with a 2702 thermoautosampler, a binary gradient pump and a 2998 photodiode array detector. The sample was eluted by 0.1% TFA in water (A) and 100% acetonitrile in 0.1% TFA (B) using 2% B over 5 min, and then increased to 25% B in 45 min, to 50% B in 5 min, to 100% B in 5 min, and to 2% B in 5 min and stay at 2% B condition for another 5 min at a flow rate of 3.0 mL/min. A total of 16 fractions were eluted and detected at 220 nm and the

Table 1
Proteolytic activity of enzymes used, optimum digestion conditions, yield and ORAC-FL value of different egg white hydrolysates.*

Enzyme	Proteolytic activity of enzyme#	Optimum pH and temperature	Digestion yield %	ORAC (μmol TE/mg sample)	ABTS (μmol TE/mg sample)
Protease P	60,000 U/g	pH 7.5; 45 °C	93.3	1.28 ± 0.06a	1.61 ± 0.00b
Protease M	5500 U/g	pH 5.0; 45 °C	62.5	1.30 ± 0.01a	1.94 ± 0.16a
Protease S	10,000 U/g	pH 8.0; 70 °C	69.4	0.90 ± 0.04c	1.24 ± 0.09c
Protex 51FP	400,000 HU/g	pH 7.5; 50 °C	69.7	0.96 ± 0.07c	1.24 ± 0.09c
Alcalase	2.4 U/g	pH 7.5; 50 °C	75.5	1.14 ± 0.04b	1.29 ± 0.00c

[a–c]Values with different superscripts within the same column indicate significant differences at $P < 0.05$ separated by Duncan's multiple range test.

* Results are means ± Standard deviation ($n = 3$).

\# Enzyme activity was reported previously (Navidghasemizad, Acero-Lopez, Curtis, Temelli, & Wu, 2014) with the exception of activity of alcalase which was provided by the vendor (Sigma).

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