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# Purification and characterization of a natural antioxidant peptide from fertilized eggs

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ABSTRACT

Fertilized hen eggs are traditionally considered as dietary supplements in many Asian countries. This work aimed to obtain information on antioxidant peptides derived from fertilized eggs. Antioxidant activities were evaluated by measuring reducing power, DPPH radical scavenging activity and inhibition of pyrogallol autoxidation. During 15 days of incubation, the antioxidant activity of peptides increased with increasing incubation time. The peptides on day 15 were employed for isolation of antioxidant peptide. An antioxidant peptide, HLFGPPGKKDPV (MW: 1291.51 Da), was purified by consecutive chromatographic methods. The purified peptide was a novel peptide corresponding to the fragment 302–313 of ovotransferrin. The conformational prediction and Fourier transform infrared spectroscopy suggested that the peptide existed in β-sheet. Furthermore, the peptide showed an inhibition ratio of 60.20% on linoleic acid autoxidation and an inhibitory effect on ABTS radicals ($IC_{50}$: 312 μM). These results suggested that fertilized eggs could be explored as a source of antioxidant peptides.



## 1. Introduction

Reactive oxygen species (ROS) such as superoxide anion ($O^{2-}$), hydroxyl radical (•OH) and hydrogen peroxide ($H_2O_2$) are normally produced in living organisms during metabolism (Ahn, Kim, & Je, 2014). These ROS have a probable role in different disorders, such as diabetes, hypertension, atherosclerotic cardiovascular disease and cancer (Memarpoor-Yazdi, Asoodeh, & Chamani, 2012). Antioxidants can scavenge ROS and prevent the cell injury, thereby playing an important role in human health (Sheih, Wu, & Fang, 2009). As chemical antioxidants like butylated hydroxyanisole (BHA) and butylated hydroxytoluene (BHT) may pose potential health hazards, many studies have been initiated to develop novel, safe and natural antioxidants (Lee, Jeon, & Byun, 2011). In recent years, the bioactive peptides derived from food proteins have received more attention due to having safer and milder activities (Memarpoor-Yazdi et al., 2012). Several antioxidant peptides have been isolated from various protein sources, such as soy protein (Park, Lee, Baek, & Lee, 2010), casein (Suetsuna, Ukeda, & Ochi, 2000), whey protein (Peng, Kong, Xia, & Liu, 2010), gelatin (Mendis, Rajapakse, & Kim, 2005) and wheat gluten (Suetsuna & Chen, 2002).

Fertilized hen eggs are traditionally considered as natural dietary supplements in some Asian countries (Li, Su, Sun, & Yang, 2012). In early incubation, the chick embryo is exposed to considerably high oxygen tensions (Surai, 2000). Therefore, an effective antioxidant system is considered to develop with increasing incubation time (Surai, Noble, & Speake, 1996). Previous researches have been reported on some antioxidants that protected chick embryo from attack by oxygen tensions (Chay Pak Ting et al., 2011; Gaál, Mézes, Noble, Dixon, & Speake, 1995; Surai, 1999, 2000; Surai et al., 1996; Wilson, Lui, & Del Maestro, 1992). Surai et al. (1996) reported that α-tocopherol and carotenoids in the embryonic liver were far higher than in any other tissue, while the concentration of ascorbic acid in the embryonic brain was far higher than in other tissues. Surai (1999) also investigated the expression of the antioxidant enzymes, such as glutathione peroxidase (GPx), superoxide dismutase (SOD) and catalase (CAT), in different embryonic tissues during incubation. These studies indicated that different tissues of chick embryo displayed distinct development strategies with regard to the acquisition of antioxidant capacity. However, most of these researches focused on nonpeptidyl antioxidants, such as α-tocopherol, carotenoids, selenium, ascorbic acid, GPx, SOD and CAT, while the information on antioxidant peptides is still limited. To our knowledge, this is the first study to investigate the antioxidant activity of peptides derived from fertilized eggs.

The objectives of the present study were to: 1) examine the change in antioxidant activity of peptides derived from fertilized

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eggs; and 2) isolate and characterize a natural antioxidant peptide from fertilized eggs.

## 2. Materials and methods

### 2.1. Materials

Fertilized hen eggs were obtained from a local producer (Zudai Poultry Co., Wuxi, Jiangsu, China). The eggs were incubated at 37.8 °C and 60% relative humidity in a bench-top incubator (Brinsea Products, Banwell, United Kingdom). Eggs were automatically turned through an angle of 90° every hour. Fertilized eggs were obtained on days 3, 4, 5, 6, 9, 12 and 15 of incubation. On each day, representing the developmental stage studied, 10 eggs were obtained and the entire contents were homogenized in a DS-1 high-speed tissue triturator (Specimen Model Factory, Shanghai, China). The obtained samples were lyophilized in a Lyph-Lock 12 vacuum freeze drier (Labconco, Kansas City, MO) at −60 °C and 7 μm Hg vacuum for 2 days. The totally dried powders were stored under −70 °C for further analysis. The testing chemicals including 2,2-diphenyl-1-picryhydrazyl (DPPH), 2,2-azinobis (3-ethylbenzothiazoline-6-sulphonic acid) (ABTS), pyrogallic acid, and α-tocopherol were purchased from Sigma Chemical Co. (St. Louis, MO, USA). All other reagents were of analytical grade (Shanghai Chemical Reagents Company, Shanghai, China).

### 2.2. Preparation of peptides

Peptides were prepared with the method described by Xu, Cao, He, and Yang (2009). The lyophilised samples (2 g) were suspended in 100 ml of Milli-Q water and centrifuged (10,000 ×g, 20 min, 4 °C). The supernatant was microfiltered through a 0.45-μm membrane and then ultrafiltrated through a 3 kDa cellulose membrane (Amicon, Beverly, MA, USA). The filtrate was dialyzed with a nanofiltration membrane of 200 Da to remove small molecules. The obtained samples were lyophilized in a Lyph-Lock 12 vacuum freeze drier at −60 °C and 7 μm Hg vacuum for 2 days. The totally dried powders were stored under −70 °C for further analysis. The peptide concentrations in samples were measured by the biuret method of Hatefi, Haavik, and Griffiths (1962). The peptide contents of the lyophilized samples were about 93% and there was no significant difference among different samples.

### 2.3. Determination of antioxidant activity of peptides

#### 2.3.1. Reducing power

Reducing power was measured according to the method described by Klompong, Benjakul, Kantachote, and Shahidi (2007). Briefly, the peptides (10 mg) of each developmental stage were mixed with 2.5 ml of phosphate buffer (0.2 M, pH 6.6) and 2.5 ml of 1% potassium ferricyanide. Following incubation at 50 °C for 20 min, 2.5 ml of 10% trichloroacetic acid (w/v) was added. After vortexing, the solution was centrifuged at 2000 ×g for 10 min (Hettich mikro 20, Hettich, Tuttlingen, Germany). Finally, 2.5 ml of the supernatant was mixed with 2.5 ml of distilled water and 0.5 ml of 0.1% ferric chloride (w/v). Following incubation at room temperature for 10 min, the absorbance was read at 700 nm. Higher absorbance ($A_{700}$) indicated greater reducing power.

#### 2.3.2. DPPH radical scavenging activity

DPPH radical scavenging activity (RSA) was measured according to the method described by Zhang, Li, Miao, and Jiang (2011). Two milliliters of peptides (10 mg/ml in PBS buffer) of each developmental stage was mixed with 2 ml of 95% ethanol containing 0.1 mM DPPH radicals. The mixture was allowed to stand in the dark for 30 min, and the absorbance was monitored at 517 nm with a spectrophotometer (JASCO, Japan). The control was conducted in the same manner where 95% ethanol was used instead of sample. DPPH RSA was calculated as following:

$$\text{RSA}\ (\%) = \left(A_{\text{control}} - A_{\text{sample}}\right)/A_{\text{control}} \times 100,$$

where $A_{\text{sample}}$ was the absorbance of the sample and $A_{\text{control}}$ was the absorbance of the control.

#### 2.3.3. Inhibition of autoxidation of pyrogallic acid

The superoxide anion ($O^{2-}$) scavenging capability was assayed according to the method described by Xiang and Ning (2008). Briefly, mixtures of Tris–HCl buffer (4.5 ml, 50 mmol/l, pH 8.0) and sample solution (1.5 ml, 10 mg/ml peptides in PBS buffer) were incubated (25 °C, 10 min) before 300 μl of pyrogallic acid (3 mmol/l, prepared in 10 mmol/l HCl) was added. The absorbance of reaction mixture at 325 nm was measured immediately at 30-s intervals. The autoxidation rate constant ($K_b$) was calculated from the curve of $A_{325\ nm}$ vs time. The inhibitory actions of test samples against the autoxidation rate indicated their $\bullet O^{2-}$ scavenging ability.

### 2.4. Purification of antioxidant peptide

Based on antioxidant activity results, the peptides on day 15 exhibited the highest antioxidant activity, and thus were employed for isolation of antioxidant peptide. The lyophilized peptides on day 15 were dissolved in 20 mM Tris–HCl (pH 8.0) and loaded onto an anion exchange column (Mono Q 5/50 GL column, internal diameter 5 mm, length 50 mm) with the ÄKTA purifier system (Amersham Pharmacia Biotech, Uppsala, Sweden), which was equilibrated with Tris–HCl (20 mM, pH 8.0). Peptides were eluted with a linear salt gradient (0 to 1.0 M NaCl for 30 min) in the same buffer at a flow rate of 2 ml/min. All fractions were collected and lyophilized in a Lyph-Lock 12 vacuum freeze drier (Labconco, Kansas City, MO) at −60 °C and 7 μm Hg vacuum for 2 days. The totally dried powders were stored for DPPH radical scavenging assay. The most active fraction was dissolved in phosphate buffer (0.05 M sodium phosphate, 0.15 M NaCl, pH 7.0) and further fractionated by size-exclusion chromatography (Superdex Peptide 10/300GL column) at a flow rate of 0.5 ml/min. All peaks were collected and lyophilized for DPPH RSA. Finally, the lyophilized fraction having the highest antioxidant activity was subjected to reverse phase high performance liquid chromatography (RP-HPLC) on an XBridge BEH130 C18 column (4.6 × 250 mm, Waters, USA) with a linear gradient of acetonitrile (10–100%) containing 0.1% trifluoroacetic acid (TFA) at a flow rate of 1.0 ml/min. Elution peaks were detected at 220 nm.

### 2.5. Identification and synthesis of the purified peptide

The fraction with the highest antioxidant activity was characterized for identification of its amino acid sequence. LC–MS/MS analysis was performed with a LTQ mass spectrometer (Thermo Electron, Bremen, Germany) coupled with a HPLC system through a nanoelectrospray ionization source. HPLC was equipped with a reversed phase C18 column (0.15 mm × 10 cm). The MS/MS spectra were analyzed by Bioworks 3.2 software (Thermo Electron) for peptide identification.

The purified peptide was synthesized with the peptide synthesizer (Symphony, Protein Technologies Inc., Tucson, AZ, USA) by the solid phase method. [(9-Fluorenylmethyl)oxy]carbonyl (FMOC) amino acids were successively coupled in the presence of 2-(1H-benzotriazol-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate (HBTU). The synthesized peptide was used for further analysis.

### 2.6. Determination of conformation of the purified peptide

The structure of the peptide was constructed and optimized with the polypeptide builder function of the public domain web server PEP-