Antioxidant activities and protective effects of duck embryo peptides against H_2O_2-induced oxidative damage in HepG2 cells

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ABSTRACT Previous work showed that peptides from duck eggs incubated for 15 D presented high total antioxidant activities. Here, this work explore the antioxidant activities of different segments, ZT1 (≤3 KD), ZT2 (≤10 KD), and ZT3 (≤30 KD), derived from duck embryo peptides and their protective effects against H_2O_2-induced oxidative damage in HepG2 cells. Peptides present no cytotoxicity to HepG2 cells. Moreover, ZT1 exhibits a higher ability to scavenge several radicals as well as stronger inhibition of H_2O_2-induced oxidative stress than ZT2 and ZT3. The activities of catalase and glutathione peroxidase as well as total superoxide dismutase increase in a concentration-dependent manner. Peptides are isolated from ZT1 and then subjected to LC-MS/MS to identify their sequences, followed by functional annotation, bioinformatics prediction, and hot-spot motif recognition. As a result, 413 potential functional peptides are identified, with some compounds exhibiting more than 1 function. This work will help for exploring bioactive substances in duck embryo eggs and enhance the utilization value of duck or other poultry eggs.

Key words: duck embryo, antioxidant peptide, HepG2 cell

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INTRODUCTION

Free radicals, due to unpaired electrons, are active and unstable in nature, often leading to chain reactions that can lead to their easy oxidation by other substances to form new free radicals or oxides (Viña et al., 2018). Under normal conditions, the production and removal of reactive oxygen species (**ROS**) in vivo are in a dynamic equilibrium state, and they are not harmful to the human body. However, when oxygen free radicals are overproduced, the antioxidant defense system and dynamic balance of oxygen free radicals can be destroyed. The accumulation of ROS can lead to oxidative stress, resulting in lipid peroxidation of the cellular membrane, degradation of proteins and enzymes, (Wojtunikkulesza et al., 2016), and destruction of nucleic acids, which eventually causes a variety of diseases, such as diabetes, aging, cardiovascular disease, and cancer (Khaper et al., 2017).

Antioxidants can help capture and neutralize free radicals and inhibit oxidative diffusion to improve the ability of tissues to avoid oxidative damage and prevent diseases caused by free radicals. They can be divided into natural antioxidants and synthetic antioxidants according to their source. The representative synthetic antioxidants are butyl hydroxyl anisole, butylated hydroxytoluene, terbutyl hydroquinone, etc. (André et al., 2010). Natural antioxidants derived from fruits, vegetables, and plants have been widely used in the food industry for a long time. While showing potential nutritional value, they also avoid the toxicity and side effects of synthetic antioxidants; therefore, the search for natural antioxidants has become one of the hottest topics in food science (Nunes et al., 2016).

Bioactive peptides promote many functions of metabolism and physiological regulation, such as promoting digestion and absorption, improving immunity, hormone regulation, antibacterial effects, antiviral effects, lowering blood pressure, softening blood vessels, and delaying senility (Bartesaghi et al., 2017). As a type of bioactive peptide, antioxidant peptides can reduce oxygen free radicals and hydroxyl radicals to achieve antiaging effects and to prevent the oxidation of oils and fats. Antioxidant peptides are essentially proteins or hydrolysates of proteins, most of which are derived from natural products, particularly, food proteins. Due to their strong antioxidant activity and safety, antioxidant peptides are widely used in recommended food industries and medical products. Embryo eggs are fertilized eggs that are in the process of incubation but have not yet broken the shell. Many studies showed that duck embryo eggs can improve gastrointestinal

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function, antioxidation effects, and antiaging effects, and they can produce a large number of new nutrients, such as peptides (Ma et al., 2009; Wang et al., 2010). Some studies characterized antioxidant peptides from the hydrolysates of duck egg proteins/peptides. However, there is no detailed study of the mechanism of action and the detailed characteristics of the peptidomic repertoire. We previously studied changes in the antioxidant substances in duck embryo eggs during incubation, showing that the duck embryos exhibit the strongest antioxidant activity on the 15th day during the incubation period of 0 to 24 D (unpublished data).

The aim is to extensively evaluate the antioxidant activities and protective effects of duck embryo peptides against H_2O_2-induced oxidative damage in HepG2 cells. Using LC-MS/MS to determine the primary sequences of the peptide segments ($\leq$3 KD) and explore the characteristics of the peptidomic repertoire as well as their potential biological functions by database annotation and bioinformatics prediction tools. During the onset of avian flu, many unincubated fertilized eggs did not flow smoothly into the market, causing huge economic losses. This work can provide a possible solution to this question and accelerate a potential economic growth via the manufacture of new duck embryo egg products. It is of great significance to understand the composition of duck embryo peptides and to promote the further discovery and application of products based on duck embryo peptides.

MATERIALS AND METHODS

Chemicals

2′,7′-Dichlorodihydrofluorescein diacetate (**DCFH-DA**) fluorescent probes were purchased from the Sigma Company (St. Louis, MO). HepG2 cells were obtained from the Chongqing Academy of Animal Sciences. Dulbecco's modified Eagle's medium (DMEM medium), 25% trypsin and dimethyl sulfoxide (cell culture level), fetal bovine serum (**FBS**) and dimethyl sulphoxide (**DMSO**) were purchased from GIBCO BRL Life Technology (China, Shanghai). Total superoxide dismutase (**T-SOD**), glutathione peroxidase (**GSH-px**), and catalase (**CAT**) test kits were bought from Nanjing Jiancheng Biotechnology (China, Nanjing). MTT single solution cell proliferation assay kits were obtained from Promega (China, Beijing). Additional chemicals were of analytical grade.

Preparation of the Freeze-Dried Powder of Duck Embryo Peptides

Fertilized healthy duck eggs were incubated in an incubator at 38.5°C with a relative humidity of 72%, and they were turned once every 2 h. After being incubated until 15 D, the duck embryo eggs were taken out and placed in a 4°C refrigerator. After the duck embryo eggs were disinfected on the surface, the egg shell was removed. Next, the duck embryo eggs were homogenized with a tissue homogenizer at 4°C, poured into the tray and placed into a freezing centrifuge to obtain the freeze-dried powder of the peptide mixture. Then, 5 times the volume of petroleum ether was added to the powder by degreasing several times, and 0.9% physiological saline was added to the powder to be redissolved at a concentration of 10 g/100 mL with even stirring, followed by centrifugation at 4,000 r/min for 10 min at 4°C. The supernatant was collected after centrifugation to carry out ultrafiltration separation to achieve peptides with different molecular weights after treatment by freeze-drying.

In Vitro Determination of the Antioxidant Capacity

A total of 10 mg of duck embryo peptides was dissolved in 10 mL of pure water, and then, 10 mL of 99.9% ethanol was added to form 5 mg/mL 50% ethanol peptide solution. A total of 3 mL of peptide ethanol solution was added into 2 mL of 0.01 mmol/L diphenyl picryl hydrazinyl (**DPPH**) solution. Then, the mixed solution was placed in darkness for 30 min. The absorption was determined at a wavelength of 517 nm by an ultra-violet−visible (UV−Vis) spectrophotometer. The blank group was 50% ethanol solution, and the control group was 3 mL of 50% ethanol with 1 mL of DPPH solution. An assay kit was used to determine to the scavenging ability of hydroxyl radicals, superoxide anions and the total antioxidant activity.

$$\text{Clearance of DPPH\%} = \left(1 - \frac{A_{\text{sample}} - A_{\text{blank}}}{A_{\text{congtrol}} - A_{\text{blank}}}\right) \times 100\% \quad (1)$$

Cytotoxicity Test of Duck Embryo Peptides on HepG2 Cells

HepG2 cells were cultured at 37°C in a 5% CO_2 incubator with 10% FBS and 2% penicillin G and streptomycin in DMEM medium. The HepG2 cells with a density of 5.0×10^4 cells/mL were added into a 96-well plate with 90 μL per well. Both the control group (90 μL of cell suspension + 10 μL of culture medium) and the experimental group (90 μL of cell suspension) were cultured for 24 h. Then, 10 μL aliquots of 2, 4, 6, 8, and 10 mg/mL peptide solutions were added to the experimental groups, respectively. The cell survival rate was measured by the MTT method after incubation for 24 h with 5% CO_2 at 37°C (Gerlier and Thomasset, 1986).

Determination of the Protective Effects of Duck Embryo Peptides on HepG2 Cells

The blank group (100 μL of culture medium), the control group (80 μL of cell suspension + 20 μL of

culture medium), the injury group (80 μL of cell suspension), and the protective group (80 μL of cell suspension) were set up, respectively. The HepG2 cells with a density of 6.0×10^4 cells/mL were inoculated in a 96-well plate, which was placed in a cell incubator with 5% CO_2 for 24 h at 37°C. The injured group was incubated with 10 μL of culture medium for 24 h, while 10 μL of peptide solution was added into the protective group for 24 h. Next, 10 μL of H_2O_2 with a final concentration of 400 μmol/L was added into the injured group and protective group, respectively. After incubation for 2 h, the survival rate of the cells was measured by the MTT method.

Determination of ROS in HepG2 Cells

The HepG2 cells with a density of 5.0×10^4 cells/mL were incubated in a 24-well plate for 24 h at 37°C. The protective group was incubated with 100 μL of peptide solution at a final concentration of 1 mg/mL, 5 mg/mL or 10 mg/mL. Meanwhile, the injury group was incubated in 100 μL of culture medium for 24 h. Meanwhile, the injured group and the protective group were incubated with 100 μL of H_2O_2 solution at a final concentration of 400 μmol/L for 2 h. Then, 100 μL of DCFH-DA solution (dissolved in 50 μmol/L DMSO) was added. The cell culture dish was incubated for 30 min with a 5% CO_2 concentration at 37°C at an excitation wavelength of 485 nm. The fluorescence intensity of each hole was detected by a multifunctional enzyme-labeled instrument under the emission wavelength of 525 nm.

Determination of T-SOD, CAT, and GSH-px in HepG2 Cells

A total of 2 mL of HepG2 cells with a density of 1.0×10^6 cells/mL was added into each well in a 6-well plate. The protective group was treated with 250 μL of peptide solution with a concentration of 1.0, 5.0, or 10 mg/mL. Meanwhile, the injury group was treated with 250 μL of culture medium. The injured group and the protective group were incubated for 2 h, and then, 250 μL of H_2O_2 (the final concentration was 400 μmol/L) was added. The culture solution was removed, and the cells were washed twice with PBS. The cell lysate was placed on ice for 30 min. The cells were scraped with a scraper and then centrifuged at 1,200 r/min for 10 min at 4°C. The cell suspensions were collected for 10 min at 4°C. The activities of these enzymes were measured by a bicinchonininc acid protein content kit and a GSH-px kit, respectively.

Characterization of Peptide Sequences

LC-MS/MS Analysis The freeze-dried powder of the peptides is reduced by dithiothreitol, then alkylated with iodoacetamide, digested with trypsin at 37°C for 16 h and desalinated. The peptides were removed using a high urea concentration by a C18 column and cryodesiccated before the MS test. Mobile phase A consisted of 0.1% formic acid in water solution, and mobile phase B consisted of 0.1% formic acid in acetonitrile solution. LC with a flow rate of 300 nL/min was applied. A Q-exactive HF-X mass spectrometer was operated in a positive polarity mode at 320°C. The full MS scan resolution was set at 60,000 with an AGC target value of 3e6 for a scan range of 350 to 1,500 m/z. A data-dependent top 40 method was operated during the collection of the HCD spectra. The main parameters were obtained at 15,000 MS^2 resolution with an AGC target of 1e5 and maximum IT of 45, 1.6 m/z isolation window, NCE of 27, and dynamic exclusion of 60 s. The data were directly imported into Proteome Discoverer 2.2 software for database retrieval and quantitative analysis of the peptides and proteins (Niklaas et al., 2011). To improve the quality of the analysis and reduce the false positive rate, the Proteome Discoverer 2.2 software was further used to filter the retrieval results; peptide spectrum matches (**PSMs**) with a credibility over 95% are called credible PSMs. The proteins containing at least one unique peptide (specific peptide) were trusted proteins. We only retained the trusted peptides and proteins and carried out false discovery rate (**FDR**) validation to remove the peptides and proteins whose FDR is greater than 5%. PEAKS Studio 8.0 (BSI, Canada) was used to analyze the LC-MS-MS data by using the database Sus scrofa (Lee et al., 2015).

Bioinformatics Analysis The gene ontology (**GO**) database was used to annotate the functions of the identified peptides (Ye et al., 2006). GO-based annotation is the analysis of identified proteins using InterProScan software, which involves the search of 6 well-known databases (Pfam, PRINTS, ProDom, SMART, ProSite, and PANTHER) so that the annotation results will be comprehensive. The identified peptides/proteins were subjected to the in silico analysis of their potential functional activities based on peptide sequences, physicochemical properties, and quantitative conformational relationships by support vector machine (**SVM**) models as follows: (i) Toxinpred for the prediction and design of toxic/non-toxic peptides (Gupta et al., 2013), (ii) AntiCP for the prediction of anticancer peptides (Rönnelid et al., 2005), (iii) AHTpin for the prediction of efficient antihypertensive peptides (Kumar et al., 2015), (iv) AntiInflame for the prediction of antiinflammatory epitopes (Gupta et al., 2017a), (v) CAMPR3 for the prediction of antimicrobial peptides (Waghu et al., 2016), (vi) dPABBs for the prediction of peptides against bacterial biofilms (Sharma et al., 2016a), and (vii) AVPpred for the prediction of antiviral peptides (Thakur et al., 2012).

Statistical Analysis

All experiments were performed in triplicate. All data are expressed as the mean ± SD. Means were compared

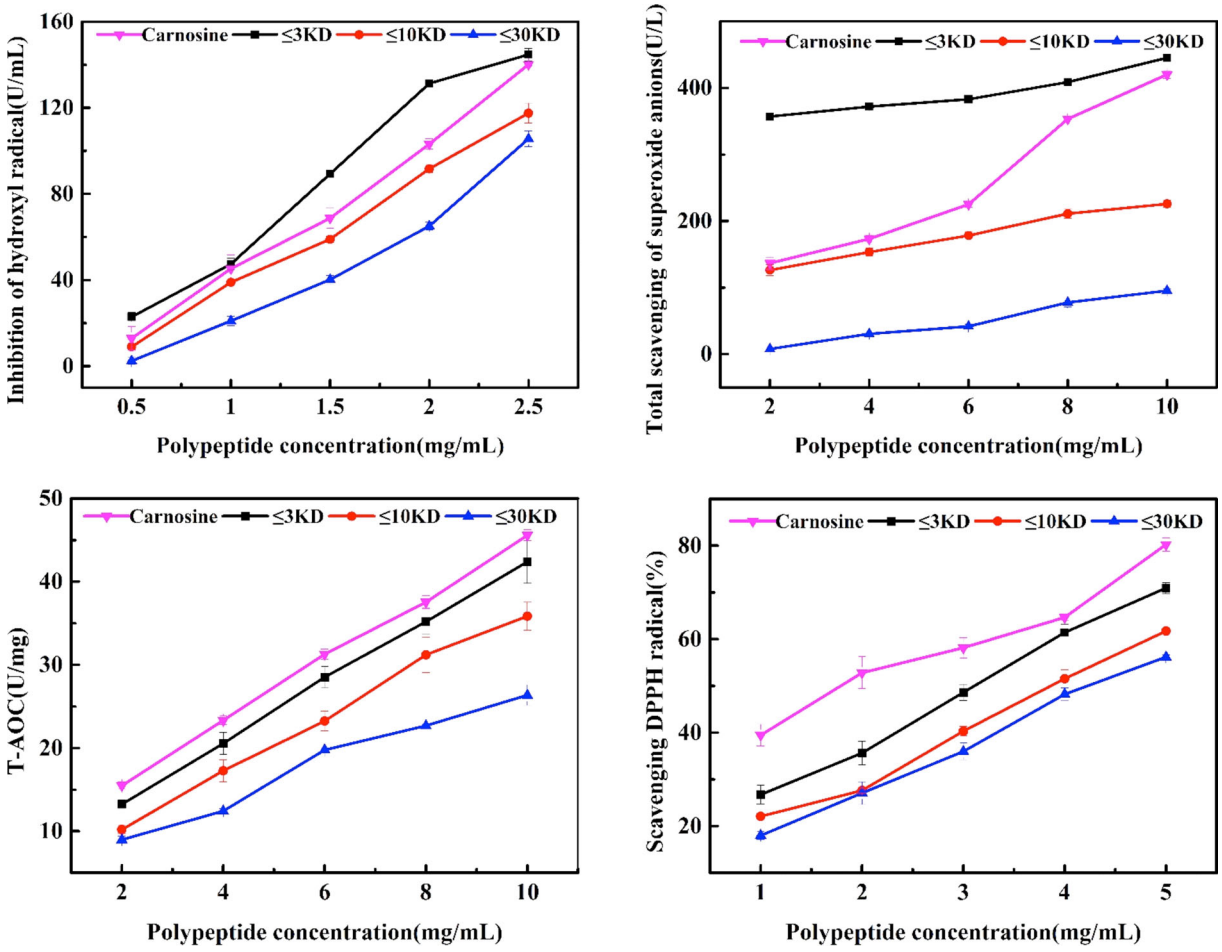

Figure 1. Antioxidant capacity of duck embryo peptides in vitro.

by one-way analysis of variance, and the level of confidence required for significance was set at $p < 0.05$. All calculations were performed by using SPSS 19.0 software.

RESULTS

Antioxidant Capacity of Duck Embryo Peptides In Vitro

This work determined the in vitro antioxidant abilities of duck embryo peptides with different molecular weights (ZT1 (≤3 KD), ZT2 (≤10 KD), and ZT3 (≤30 KD)) using 4 antioxidant assay experiments, including scavenging DPPH free radicals, scavenging superoxide anions, inhibition of hydroxyl radicals, and total antioxidant capacity. As displayed in Figure 1, ZT1, ZT2, and ZT3 all present strong antioxidant activities by all 4 methods; particularly, the antioxidant activity of ZT1 is the strongest. Moreover, with the increase of the peptide concentration, the antioxidant activity increased by varying degrees, indicating that the antioxidant capabilities were all concentration-dependent. It should be noted when the concentration of the ZT1 peptides was 2 mg/mL, the scavenging ability of superoxide anions was 356.84 U/g, whereas 10 mg/mL ZT2 and ZT3 only exhibited scavenging abilities of 225.84 U/g and 95.44 U/g, respectively, indicating that the scavenging ability of the superoxide anion of ZT1 was much higher than those of ZT2 and ZT3. Carnosine is a commercial antioxidant peptide that has a significant inhibitory effect on lipid oxidation caused by free radicals and metal ions (Ruzza, 2005). The antioxidant capacity of carnosine in vitro was measured to compare with that of the duck embryo peptides. This work showed that the total antioxidant capacity and the effect of scavenging DPPH free radicals of duck embryo peptides were lower than that of carnosine, whereas the ability to scavenge hydroxyl radicals and superoxide anions of ZT1 was higher than that of carnosine. The reasons that carnosine and ZT1 exhibited different the ability for scavenging different free radicals need to be further explored using various experimental and computational methods such as quantum-chemical calculations and western blotting.

Cytotoxicity of Duck Embryo Peptides on HepG2 Cells

To eliminate the nonspecific interference, this work detected the effects of duck embryo peptides on the

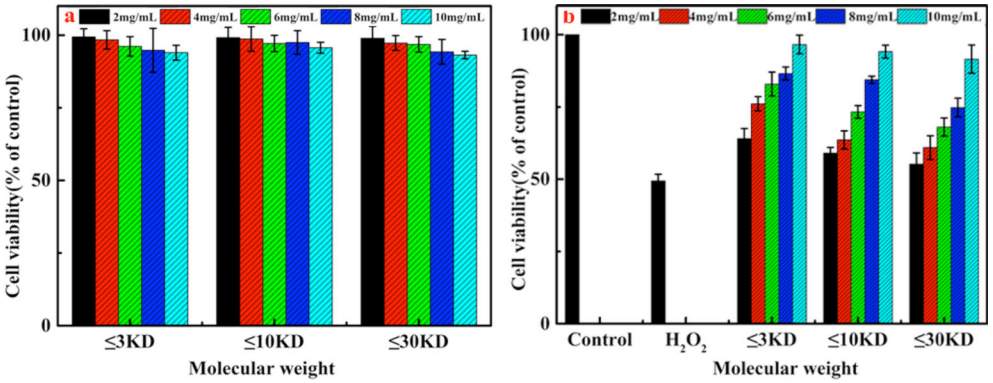

Figure 2. Toxic effects of duck embryo peptides on normal HepG2 cells (a) and protective effects of duck embryo peptides on H_2O_2-induced oxidative damage in HepG2 cells (b).

growth of HepG2 cells by the MTT method. Figure 2a shows that there is a slight difference in the cell survival rate between the experimental group and the control group, suggesting that the peptides take on weak cytotoxicity towards HepG2 cells.

Protective Effects of Duck Embryo Peptides on the H_2O_2-Induced Oxidative Damage of HepG2 Cells

H_2O_2, as a kind of ROS, can induce lipid peroxidation and enhance oxidative stress, which can finally lead to cell damage or even death. The H_2O_2-induced oxidative destruction of cells has been widely used to evaluate the treatment of oxidative stress by many bioactive substances. The HepG2 cells are often used to establish different antioxidant models to study the antioxidant capacities of the substances. Thus, this work evaluated the protective effects of the peptides on the H_2O_2-induced oxidative damage of HepG2 cells. Figure 2b displays that ZT1, ZT2, and ZT3 inhibited H_2O_2-induced oxidative stress. The cell survival rate of the injured group treated with only H_2O_2 was 49.9% that of the control group. In the experimental group, with the increase of the peptide concentration, both the ability to inhibit oxidative stress and the protective effect from oxidative damage to the cells were enhanced, indicating that the protective effects of ZT1, ZT2, and ZT3 on cell survival were all concentration-dependent. Especially, when the concentration of ZT1 was 10 mg/mL, the survival rate (96.6%) was only slightly lower than that of the control group. In combination with the cytotoxicity test, this work proved that the protective effects of duck embryo peptides on HepG2 cells were achieved by inhibiting oxidative stress rather than by stimulating the proliferation of HepG2 cells.

Inhibitory Action of the H_2O_2-Induced Damage by ROS of HepG2 Cells

DCFH-DA, without any fluorescence itself, is a kind of fluorescent dye which can freely penetrate the cell membrane. Intracellular ROS oxidize non-fluorescent DCFH-DA to fluorescent DCF. By detecting the fluorescence intensity of DCF, the level of ROS in cells can be obtained. In fluorescence photos, the stronger the fluorescence intensity is, the higher the ROS content in the cells. Here, this paper used the DCFH-DA fluorescence probe to detect the intracellular ROS level. The fluorescence intensity of DCF in HepG2 cells was significantly enhanced after treatment with H_2O_2, indicating that H_2O_2-induced ROS were produced in HepG2 cells. In the current study, H_2O_2 induced a rapid ROS improvement in HepG2 cells within 2 h, leading to a 6.79-fold increase in the DCF fluorescence density compared to the control group. The level of ROS of the pretreated peptide group was significantly lower than that of the injured group ($p < 0.01$) in a concentration-dependent manner (Figure 3l). Specifically when ZT1 was 1 mg/mL, the relative fluorescence intensity was only 29.31 (Figure 3e), which was lower than 41.89 for the 10 mg/mL ZT3 pretreated group (Figure 3i). Results demonstrated that the peptides isolated from duck embryos could inhibit the damage of ROS to HepG2 cells.

Enhancement of the Content of Oxidases in HepG2 Cells

T-SOD, CAT, and GSH-px are important components in enzymes in the cellular antioxidant defense system. They can produce very distinct influences on the dynamic balance of oxidation and oxidation resistance in the body. The activities of T-SOD, CAT, and GSH-px significantly decreased in HepG2 cells treated with H_2O_2, whereas their activities increased with the increase of the peptide concentration in the protective group treated with ZT1, ZT2, and ZT3 (Table 1). The activities (U/mgprot) were 29.79, 26.19, and 24.81 for T-SOD, 19.78, 18.61, and 17.61 for CAT, and 432.57, 177.22, and 100.03 for GSH-px in HepG2 cells treated with ZT1, ZT2, and ZT3 at a concentration of 1.0 mg/mL, respectively.

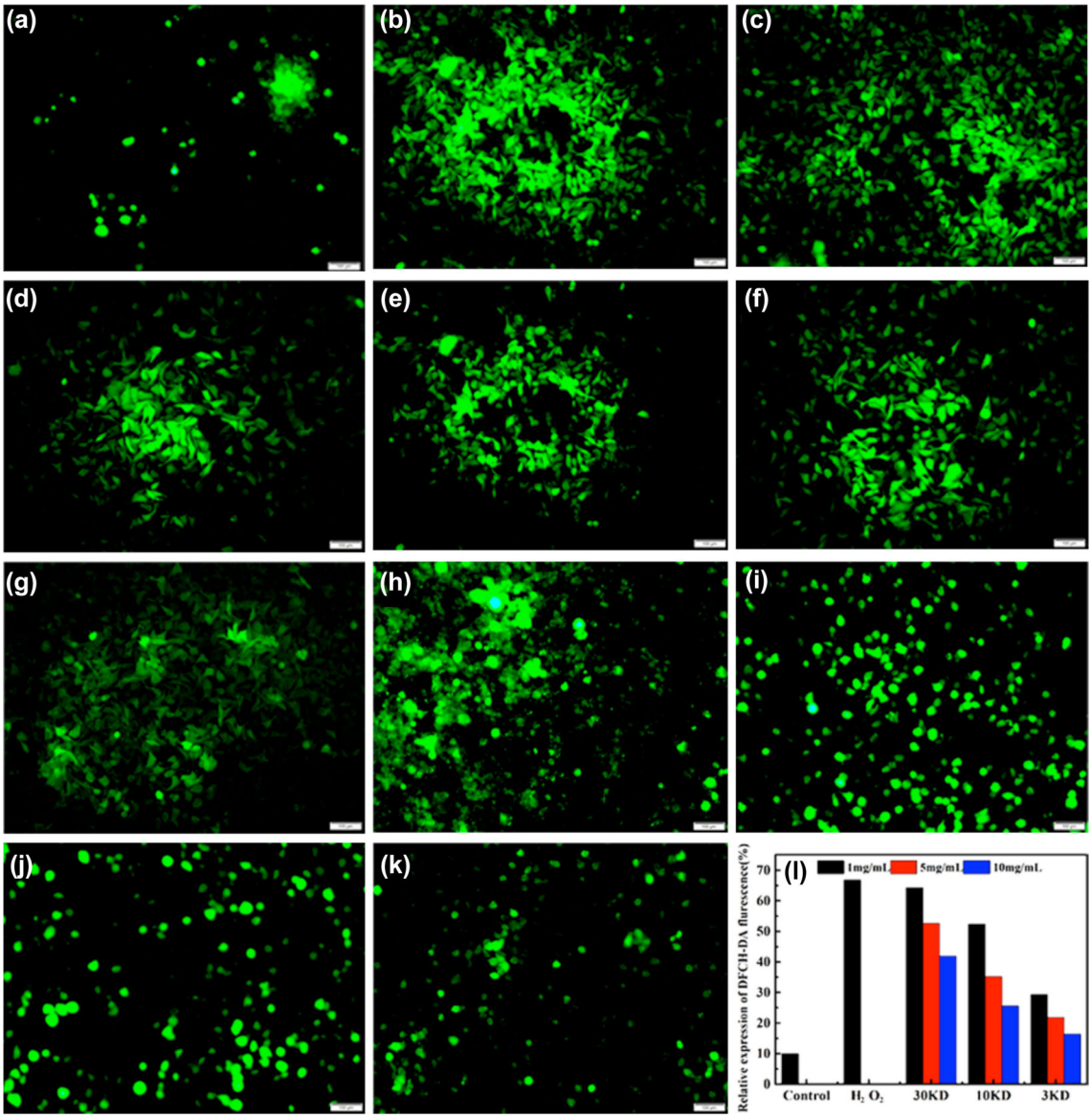

Figure 3. Fluorescence images of DCF in HepG2 cells. (a) Control; (b) H_2O_2-treated; (c) 1 mg/mL 30 KD+H_2O_2; (d) 1 mg/mL 10 KD +H_2O_2; (e) 1 mg/mL 3 KD +H_2O_2; (f) 5 mg/mL 30 KD +H_2O_2; (g) 5 mg/mL 10 KD +H_2O_2; (h) 5 mg/mL 3 KD+H_2O_2; (i) 10 mg/mL 30 KD+H_2O_2; (j) 10 mg/mL 10 KD+H_2O_2; (k) 10 mg/mL 3 KD+H_2O_2; and (l) relative expression of DCFH-DA fluorescence in different groups.

Molecular Composition of Crude Peptides Annotated by the GO Database

The duck embryo peptides in the less than 3 KD fraction presented strong oxidation activities. Thus, after desalting, these peptides were injected into LC-MS/MS for the determination of peptide sequences. A total of 919 peptides from duck embryo eggs were detected (the molecular weight distribution of all of the 919 peptides is shown in Figure S1). By GO-based annotation, we observed that these peptides were derived from 102 proteins. The biological processes associated with these proteins mainly included signal transduction, microtubule-based processes, lipid transport, proteolysis, and oxidation−reduction processes. There were 58 proteins related to cellular components, the 2 most abundant of which were 12 proteins associated with the extracellular region and 11 proteins associated with the extracellular space. There were 101 proteins concerned with molecular function, of which 20 were associated with protein binding, 10 with GTP binding, and 8 with nucleic acid binding (Figure 4). The same protein presented multiple potential functions, i.e., the U3J6P0 protein may be involved in molecular function (endopeptidase inhibitor activity and protein binding), cellular components (extracellular region and extracellular space), and biological processes (inflammatory response and complement activation). The A0A1V0ENN2 protein may affect molecular function (vitamin D binding and vitamin transporter activity), cellular components (extracellular space), and biological processes (transport and vitamin transport).

Table 1. Changes in the activities of T-SOD, CAT, and GSH-px in HepG2 cells.[a]

Groups	Concentration (mg/mL)	T-SOD (U/mg)	CAT (U/mg)	GSH-px (U/mg)
Control		50.29 ± 0.0028	38.12 ± 0.0030	518.21 ± 0.0021
H_2O_2		21.93 ± 0.0120	14.61 ± 0.0020	89.02 ± 0.0033
≤30 KD	1	24.81 ± 0.0002	17.61 ± 0.0021	100.03 ± 0.0014
	5	28.32 ± 0.0011	21.34 ± 0.0013	178.21 ± 0.0018
	10	30.64 ± 0.0001	24.29 ± 0.0016	225.97 ± 0.0013
≤10 KD	1	26.19 ± 0.0021	18.61 ± 0.0018	177.22 ± 0.0057
	5	29.12 ± 0.0120	24.21 ± 0.0032	267.32 ± 0.0015
	10	31.86 ± 0.0032	26.32 ± 0.0027	343.89 ± 0.0022
≤3 KD	1	29.79 ± 0.0041	19.78 ± 0.0044	228.51 ± 0.0031
	5	33.54 ± 0.0022	27.13 ± 0.0013	372.98 ± 0.0020
	10	36.62 ± 0.0001	32.39 ± 0.0032	432.57 ± 0.0053

[a]The table shows mean values ± SD (n = 3).

Antioxidant Peptides Annotated by the GO Database

A total of 5 antioxidant peptides were determined by hydrolysis from glyceraldehyde-3-phosphate dehydrogenase (**GAPDH**), sulfhydryl oxidase and an uncharacterized protein by GO annotation. A total of 3 antioxidant peptides, VIHDNFGIVEGLMTTVHAITATQK, TVDGPSGKLWRD, and GAAQNIIPASTGAAKAV, were derived from hydrolysis at different protein loci in GAPDH, which is considered to be a glycolytic enzyme involved in various compression reactions. The remaining 2 identified fragments, RLAGGDTEDPQFPK and LVDQLVDPLGPGVKTPEA, were derived from a sulfhydryl oxidase and an uncharacterized protein, respectively.

Functions of the Peptides Predicted by SVM

To expand the scientific application of these peptides, this paper extensively analyzed these peptides through a variety of web-based prediction tools and database-based functional screening. A total of 413 peptides (Table S1) were predicted to at least contain putative biological activity, while the remaining 506 peptides were predicted to not have any potential function (Table S2). Of these 413 proteins, there were 132 antioxidant, 48 anticancer, 135 antihypertensive, 50 antiinflammatory, 16 antibiofilm, 78 antimicrobial, and 38 antiviral peptides, with some compounds exhibiting more than one function. It is worth noting that 5 peptides exhibited potential toxicity, thus further utilization of these peptides required caution.

Hot-Spot Antioxidant Motifs

This work shed light on the characteristics of the 132 predicted antioxidant peptides, which included the 5 antioxidant peptides derived from GO annotation as mentioned above. These antioxidant peptides contained different antioxidant motifs that included at least 2 of the following amino acids: Cys, Gly, His, Val, Ala, Leu, Arg, Tyr, Trp, and Phe, which were reported to be associated with antioxidant activity. Particularly, the above amino acids sequences of ALAPLAIPSAAAAAAAAGR and GALAPLAIPSAAAAAAAAGR, both derived from U3IEZ5 (332-351) (333-351), accounted for 79% and 80% of all of the amino acids, respectively. In agreement with previous studies on antioxidant peptides from foods, analogous cysteine- or aromatic amino acid-rich di-, tri-, and tetrapeptides as well as other motifs elicit a putative protective action on the antioxidant system. The present peptides included (i) 1 GAPDH peptide in a portion (185−196) containing the WR motif, (ii) 1 U3IDX1 peptide in a portion (13−31) containing the LW motif (Klassen et al., 2000), (iii) 29 peptides with the AG motif (Garcia-Redondo et al., 2010), (iv) 19 peptides (10 from APO, 6 from Anapl and 3 from U3IDX1) with the WE motif (Dave et al., 2016a), (v) 37 peptides (12 from Apovitellenin-1 and others from different proteins) with the AQ motif (Wang et al., 2012), (vi) 15 peptides (13 from Anapl) with the TY motif (Liu et al., 2015), (vii) 34 peptides (mostly from Anapl_07192) with the TF motif (Dave et al., 2016a), (viii) 29 peptides including the di-, tri- and tetra-peptide motifs with the MET residue (Stadtman et al., 2005), (ix) 14 peptides including the di-, tri- and tetra-peptide motifs with the His residue (Seo et al., 2010), and (x) 26 peptides including the tripeptide motif with the TRP residue, which present favorable antioxidant activities (Tian et al., 2015).

Assisted Annotations by COG, KEGG, and IPR

In addition to the method based on GO annotation, three databases/methods, Kyoto Encyclopedia of Genes (**KEGG**) and Clusters of Orthologous Groups (**COG**) and InterProScan, are often used to annotate and understand the functional properties of proteins. The InterPro (**IPR**) database was used to annotate unknown proteins by InterProScan software adopting schema structures or features to annotate their domains, while both KEGG and COG annotation could identify proteins by BLAST-based sequence alignment. Here, utilizing the three databases/methods to explore

Figure 4. GO annotation results of proteins (the results of only the top 20 in each large class are shown).

the functions of the peptides/proteins. Identified 47, 124, and 122 functional proteins by using the COG (Figure S2), KEGG (Figure S3), and IPR (Figure S4) annotations, respectively. Founded that a total of 46 functional proteins (Figure 5) were identified by the 3 databases/methods (COG, KEGG, and IPR) as well as GO annotation. The 46 proteins (Table S3) originate from Anas platyrhynchos. Of the 47 proteins annotated by COG, only the A6ZIB9 protein, as an actin without any function in biological processes, was not identified by both the KEGG and IPR methods. Of the 46 proteins identified by the 3 databases/methods, the 3 kinds of most abundant proteins are 11 proteins related to translation, ribosomal structure, and biogenesis,

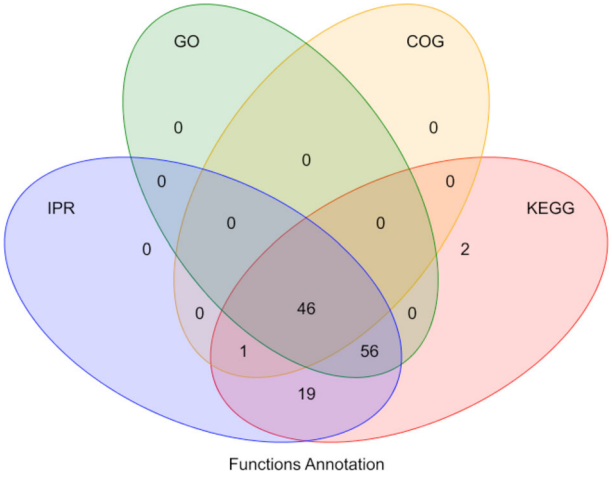

Figure 5. Statistical results of functional proteins annotated by the 4 databases/methods.

10 proteins related to post-translational modification, protein turnover, and chaperones, and 6 proteins related to carbohydrate transport and metabolism including oxidative stress (Kwon et al., 2009), respectively.

DISSCUSION

The antioxidant protein database found that antioxidant proteins are enriched in residues including Cys, Gly, His, and Val (Feng et al., 2017). In addition, it has been reported that several residues, Ala, Leu, Arg, Tyr, Trp, and Phe, were important for the antioxidant activity of the peptides (Zheng et al., 2016). The amino acid compositions of the 5 antioxidant peptides were over 42% of the amino acids mentioned above. Particularly, TVDGPSGKLWRD, GAAQNIIPASTGAAKAV, and LVDQLVDPLGPGVKTPEA accounted for more than 50% of all of the amino acids. In accordance with previous studies on antioxidant peptides from eggs or other sources, a structural composition rich in aromatic amino acids with 2, 3, and 4 peptide motifs could protect ROS-mediated oxidation. Here, 13 U3IWA4 (Uniprot protein access number) peptides from regions (4,556 to 4,575), 10 R0KDM8 fragments from regions (1,049 to 1,070) and others containing the WE, WK, WR, TF, YF, TY, MY, CS, AQ, AG, and LM motifs, including aromatic or sulfur-containing amino acids (Table S1), were identified, which were already identified in other antioxidant peptides from food hydrolysates.

We were also interested in the characteristics of the other peptides by the SVM-based methods. Studies have shown that hydrolysates of egg yolk and livetin exhibited anticancer functions. Of the 48 anticancer peptides identified here by AntiCP scanning, 12 came from APOA1 fragments from regions (44 to 1,144), which has been found to inhibit tumor development in mouse models of ovarian and colon cancer (Feng et al., 2010). It has been reported that these peptides occurred in a protein helical portion corresponding to peptide mimetic products (Feng et al., 2010). A total of 2 peptides, U3IWA4 (3,733 to 3,746) and R0LGD6 (1,544 to 1,557), contained the KA or FR motifs, which often appeared in antihypertensive peptides (Garcia-Redondo et al., 2010). The predicted antihypertensive peptides were mostly derived from fibrinogen alpha chain and tubulin alpha chain containing YP, QK, or KP motifs, which were already seen in peptide-based ACE-I inhibitors (Dave et al., 2016b). All of the predicted antiinflammatory peptides involved the L, S, Y, and R residues as well as hydrophobic and polar amino acids, which played an important role in the antiinflammatory effect of peptides (Gupta et al., 2017b). These antiinflammatory peptides mainly came from Vitellogenin-2 protein. Studies have shown that the protein by-products/peptides extracted from egg yolk contain a large amount of Vitellogenin-2 protein and can be converted into value-added products, such as potential health care enhancement antioxidants, with antiinflammatory and antihypertensive effects (Yousr and Howell, 2015). Eggs contain a variety of proteins with antimicrobial activity, including lysozyme and o-transferrin, as well as natural defense peptides (in the eggshell, egg white, and yolk). No data have been reported on antimicrobial molecules in duck embryo eggs to date. This paper screened 63 antimicrobial peptides, of which 10 peptides contained DP as a motif contributing to the antimicrobial activity (Wang et al., 2016). Asp, Glu, Arg, and Lys have been reported to be associated with antibiofilm activity, largely due to their positive charges and high hydrophilic character (Sharma et al., 2016b). This work screened 15 antibiofilm peptides, each with at least the 3 amino acids (Asp, Glu, Arg, and Lys); especially, SIKKQLKEKGELEE and SIKKQLKEKGELE contained 8 and 7 related amino acids, respectively. The N-terminal and C-terminal of the peptides showed different importances to their antiviral activities (Houghten and Weber, 1995). Here, the N-terminals of the 38 antiviral peptides screened by SVM were mainly Ser, Ile, and Lys (8 Ser, 7 Ile, and 6 Lys), while the carbon-terminal amino acids were mainly Leu, Lys, and Gln (10 Leu, 7 Lys, and 7 Gln), indicating that these amino acids at the N-/C-terminals played an important role in the antiviral activity.

Food-derived proteins and peptides are important nutrients with additional biotic functions, which are extremely beneficial to human health (Walther and Sieber, 2011). This work evaluated the antioxidant activities and protective effects of duck embryo peptides against H_2O_2-induced oxidative damage in HepG2 cells. At a low concentration, ZT1 exhibits a stronger antioxidant capacity in vitro as well as stronger inhibitory effects on H_2O_2-induced oxidative stress and damage to HepG2 cells than ZT2 and ZT3, showing that ZT1 can be used as a new natural antioxidant in the food industry and medicine products. However, the exact sequences of the peptides are still unknown, and thus, further experiments are urgently needed to

determine their sequences and mechanisms of action. Additionally, the investigation of other functions of identified duck embryo peptides is also warranted. This work is beneficial to the discovery and application of bioactive peptides or other bioactive molecules derived from duck embryos or other food-derived materials. Exploring the antioxidant active ingredients in duck embryo eggs is beneficial to the study of diseases associated with oxidation in duck embryos. Moreover, screening various functional substances is conducive to the development of a series of high value-added products from poultry embryo eggs.

SUPPLEMENTARY DATA

Supplementary data are available at *Poultry Science* online.

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