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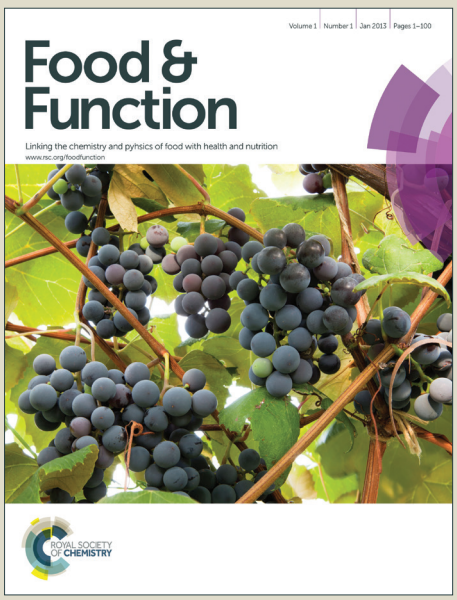

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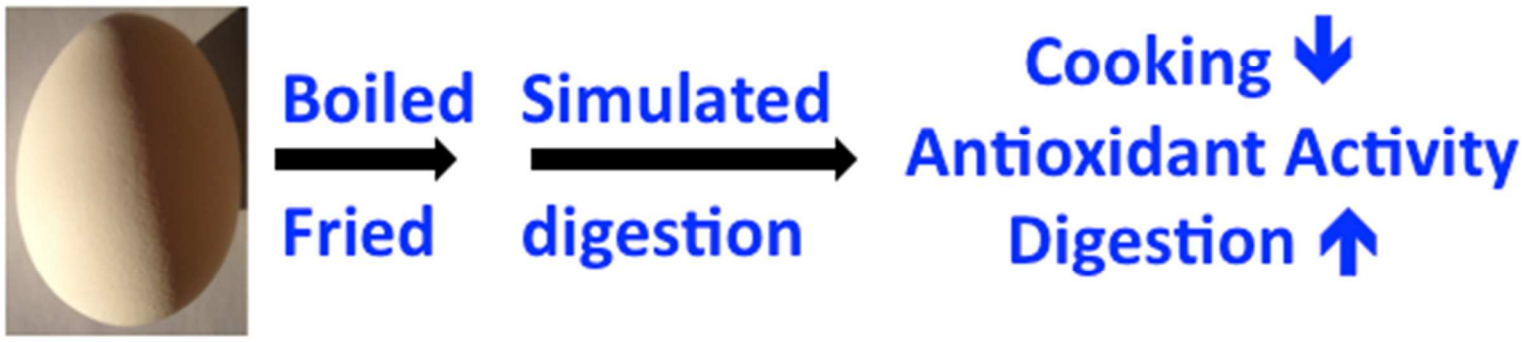

Highlights: Egg is a source of antioxidants; cooking reduces whereas digestion enhances the antioxidant activity

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ARTICLE

Antioxidant Activity in Cooked and Simulated Digested Eggs

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The avian egg is an excellent source of nutrients consisting of components with beneficial properties but there is a limited knowledge on the effect of cooking methods and gastrointestinal digestion on the antioxidant activity of eggs. The present study was focused on the effect of cooking and simulated gastrointestinal digestion on antioxidant activity of eggs using ORAC, ABTS and DPPH assays. The results suggest that fresh egg yolk have higher antioxidant activity than fresh egg white and whole eggs. Cooking reduced whereas simulated gastrointestinal digestion increased the antioxidant activity of eggs. Boiled egg white hydrolysate showed the highest antioxidant activity; a total of 63 peptides were identified, indicative of the formation of novel antioxidant peptides upon simulated gastrointestinal digestion. This study suggests the potential role of eggs as dietary source of antioxidants.

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Introduction

Oxidation of the biomolecules occurs continuously within the body due to the formation of free radicals during normal metabolic reactions involved in the respiratory chain, degradation of lipids, the catecholamine response under stress, inflammatory responses as well as from external sources such as radiations, cigarette smoking, air pollutants and industrial chemicals.[1] The free radicals formed in the body are regulated by the antioxidant defense system to maintain a balance in the redox homeostasis.[2] When free radical formation exceeds the protective capacity of the antioxidant defense system, it leads to oxidative stress which could contribute to cancer, atherosclerosis, malaria, rheumatoid arthritis and neurodegenerative diseases.[3] The antioxidant compounds can either prevent the harmful effects of free radicals or protect the biological system from the excessive damage induced by the free radicals.[4] Various endogenous antioxidants in the boas superoxide dismutase (SOD), glutathione (reduced; GSH), GSH peroxidases, glutathione reductase, catalase, as well as exogenous source of antioxidants derived from our diet, are the principal antioxidant defense systems in the body.[5, 6] Antioxidant activity from many plant food commodities and herbs has been extensively studied.[7-10] On the other hand, antioxidants from animal food commodities are less documented. Several well-known antioxidants from animal food products are carnosine[11] , milk proteins[12] and fish muscle derived peptides.[13] The avian egg is considered as an excellent dietary source of nutrients, includes proteins, lipids, vitamins, minerals, embryonic growth factors, and various components to protect from pathogens.[14] Studies on egg revealed the presence of biological components with antioxidant activities.[15] Several egg white protein, ovalbumin[16], ovotransferrin[17], lysozyme[18], phosvitin[19], were reported to have antioxidant activities. Egg yolk contains various antioxidants, such as phospholipids [19, 20], carotenoids such as lutein and zeaxanthin[19-21] , and free aromatic amino acids.[22] Cooking or food processing are known to affect antioxidants of fruits and vegetables by either increasing or decreasing the antioxidant activity.[23] Antioxidant peptides from animal proteins such as milk proteins[12], fish muscle derived peptides[13], as well as egg proteins[15, 24-28] have been extensively reported. As a protein rich food commodity, release of peptides in the human gut might further enhance the antioxidant activity and

thus impart benefits to human health. However, there is limited knowledge on the effect of cooking and gastrointestinal digestion on the antioxidant activity of eggs. Therefore, the objectives of this study were to determine the effects of cooking methods and simulated gastrointestinal digestion on the antioxidant activity of eggs.

Experimental

Materials*:* Fresh white-shell eggs were obtained from Poultry Research Centre of the University of Alberta (Edmonton, AB, Canada). Pepsin (porcine gastric mucosa) and pancreatin (porcine pancreas) were purchased from Sigma-Aldrich (Oakville, ON, Canada). Trolox (6-hydroxy-2, 5, 7, 8-tetramethylchroman-2-carboxylic acid) was obtained from Acros-Organics (Morris Plains, NJ, USA) and AAPH (2, 2'-azobis (2-amidino-propane) dihydrochloride and fluorescein (FL) (Na salt) were obtained from Aldrich (Milwankee, WI, USA). L-tryptophan was obtained from Sigma-Aldrich (Oakville, ON, Canada). Randomly methylated β-cyclodextrin (RMCD) was obtained from Cyclodextrin Technologies Development Inc. (High Springs, FL, USA).

Preparation of egg samples: For preparing fresh egg samples, egg white was separated manually from egg yolk; whole egg was prepared by homogenization after breaking. For preparing boiled eggs, fresh eggs were placed in a saucepan with water one inch above the shell and then boiled for 10 min. After boiling, the eggs were placed under running water for 5 min, peeled, and then each egg white and yolk was separated. Boiled whole eggs were prepared from homogenizing boiled egg whites and egg yolks. For preparing fried whole eggs samples, separated egg white or egg yolk and/or homogenized whole egg, were transferred to preheated frying pan (176.7^{o}C) cooked each side for 40 s. All the samples were freeze dried for further analysis.

Simulated digestion of egg samples: Freeze-dried egg samples were mixed with distilled water to make 5% slurry (w/v) and were kept in the water bath at 80°C for 15 min with continuous shaking. The temperature was adjusted to 37°C by adding ice cubes into the water bath, and the pH of the slurry was adjusted to 2 with 1 N HCl. After stabilization, pepsin (2%, w/w, pepsin/protein) was added to initiate digestion and the conditions were maintained constantly for a period of 3 h. Then the pH of the slurry was adjusted to 7.0 to inactivate the enzyme and at this point half of the sample was taken out as the pepsin digest. Pancreatin (2%) was then added to initiate another digestion of 3 h. The digestion was terminated by keeping the sample at 95°C for 15 min, and centrifuged at 10,000 x g for 25 min. The supernatant was collected, freeze dried, and stored for further analysis. The digestion was carried out using Titrando (Metrohm, Herisan, Switzerland) and a circulating water bath was used for maintaining constant temperature.

Extraction of egg yolk antioxidants: All the freeze-dried egg yolk or whole egg samples were first extracted with 10 mL of hexane/dichloromethane (1:1) with an orbital shaker for 1 h at room temperature at 600 rpm, followed by centrifugation at 3000 rpm for 5 min. The hexane/dichloromethane (H/D) layer was collected, and was evaporated under nitrogen to prepare the lipophilic fraction. The residue was dried and extracted with 80 % ethanol for 1 h with an orbital shaker at 600 rpm. The extracted samples were then centrifuged at 3000 rpm for 5 min and the supernatants were collected for hydrophilic antioxidant analysis.

Measurement of antioxidant activity: The antioxidant activity was determined using three different methods: oxygen radical absorbance capacity (ORAC) assay, 2, 2'-azino-bis (3-ethylbenzthiazoline-6-sulphonic acid) ABTS decolorization assay, and 1, 1-Diphenyl-2-picryl-hydrazyl (DPPH) assay with slight modifications as indicated below. The lipophilic extracts were dissolved in 250 µL of acetone and then diluted with 750 µL of a 7 % RMCD in 50% acetone solution (50% acetone/50% water, v/v). The 7 % RMCD acts as a water solubility enhancer for lipophilic antioxidants[29], and was used for dissolving Trolox standards, as well as used the blank. The hydrophilic extracts were prepared in phosphate buffer (75 mM, pH 7.4). All samples were extracted in duplicate and assayed in triplicate.

Oxygen radical absorbance capacity (ORAC) assay: ORAC was measured using the method explained by Davalos et al.[30], with slight modifications as we previously reported.[22] The ORAC value was calculated by dividing the slope of sample regression curve by the slope of Trolox regression curve. The final ORAC values were expressed as µmol of Trolox equivalent/mg of sample.

2, 2'-azino-bis (3-ethylbenzthiazoline-6-sulphonic acid) ABTS assay: $ABTS^{\bullet+}$ decolorization assay was based on Strljbe, Haenen, Berg, & Bast (1997) with slight modifications. ABTS radical cation was generated by mixing 7 mM ABTS and 2.45 mM potassium

persulfate and diluted 13-fold with an assay buffer (3 mM phosphate buffer at pH 7.5 containing 150 mM NaCl for hydrophilic ABTS or 95% ethanol for lipophilic ABTS) immediately before use. For each run, 20 µL of sample and 80 µL of phosphate buffer or 95% ethanol were placed in wells of a 96-well microplate, followed by addition of 100 µL of the ABTS radical solution. Absorbance was monitored at 734 nm after 5 min of incubation at 37 ^{o}C. A Trolox regression equation between absorbance and Trolox concentrations was calculated and used to calculate the Trolox equivalent antioxidant capacity (TEAC) value for all the samples. The TEAC value is expressed as µmol of Trolox equivalent/mg of sample.

1, 1-Diphenyl-2-picryl-hydrazyl (DPPH) assay: DPPH antioxidant assay was performed based on Bersuder, Hole, & Smith (1998). In brief, 20 µL of antioxidant and 80 µL of water for hydrophilic DPPH or 95% ethanol for lipophilic DPPH were placed in the wells of 96-well microplate, followed by addition of 100 µL of 0.2 mM DPPH in 95% ethanol solution. Absorbance was monitored at 517 nm after 45 min of incubation at 37 ^{o}C. A Trolox regression equation between absorbance and the standard (Trolox) concentrations was calculated and the DPPH radical scavenging activity was estimated for all the peptides. The results were expressed as µmol of Trolox equivalent/mg of sample.

Purification of antioxidant peptides from hydrolysate: The boiled egg white hydrolysate was dissolved in 10 mm ammonium acetate (pH 4) buffer and then filtered the sample by using 3000 Da ultra filtration membrane. Fractionation of permeate was performed using a HiPrep 16/10 SP FF cation exchange column (16 x 100 mm, GE Healthcare Sweden) coupled with an ÄKTA explorer 10XT system. The column was equilibrated with 10 mM ammonium acetate (pH 4) and eluted with 0.5 M ammonium carbonate buffer at a flow rate of 5 mL/min. The injection volume was 4 mL and the elution was detected at 280 nm. The most potent fraction collected in the unadsorbed fraction was further applied to HiPrep Q FF 16/10 anion exchange column (16 x 100 nm, GE Healthcare Sweden). The column was equilibrated with 10 mM ammonium acetate (pH 8.5) and eluted with 10 mM ammonium carbonate and 1 M NaCl buffer at a flow rate of 5 mL/min. The fractions exhibiting the most potent antioxidant activity was further purified by reverse-phase high performance liquid chromatography (RP-HPLC) on a Xbridge C18 column (10 mm x 150 mm, 0.5µm, Waters Inc, Milford, MA, USA) coupled with a guard column (40 x 10 mm, Waters Inc, Milford, MA, USA) attached to Waters 600 HPLC system, under the control of the software of Empower Version 2 for the instrument control and data acquisition. Sample was injected automatically by Waters 2707 autosampler at a volume of 500 µL, and was eluted using a linear gradient starting from 100% solvent A (HPLC-grade water containing 0.1% TFA) to 40 % solvent B (HPLC-grade acetonitrile with 0.1% TFA) over 40 min at a flow rate of 5 mL/min, followed by washing the column at 100% solvent B for 10 min before next run. The elution was monitored at a wavelength of 220 nm using Waters 2998 photodiode array. Fractions were collected at 2 min intervals from 3 min to 50 min (19 fractions), concentrated using vacuum-rotary evaporator at 35°C, and the antioxidant assays (ORAC, DPPH and ABTS) of each were determined.

Liquid chromatography-Tandem Mass Spectrometry (LC-MS/MS): Identification of the peptides in the most antioxidant active fractions from the RP- HPLC separation was carried out by Waters ACQUITY UPLC system connected online to Waters (Micromass) Q-TOF Premier (Milford, MA, USA). Peptides were separated by Waters Atlantis dC_{18} (75 µm x 150 mm, 3 µM) UPLC column (Milford, MA, USA). The separation was carried out using solvent A, 0.1% formic acid in optima LC/MS grade water and solvent B, 0.1% formic acid in optima grade acetonitrile. Samples in Solvent A (5 µL) was injected to the 5 µm trapping column for 2 min at a flow rate of 10 µL/min using 99% solvent A, followed by a gradient from 99% A to 90% A over 5 min, to 70% A over 30 min, to 60% A over 3 min and 5% A over 1 min at a constant flow rate of 0.350 µL/min, increased the flow rate to 0.500 µL/min and held at 5% A for 2 min, with subsequent increased to 98% A over 1 min, held for another 27 min, and then decreased the flow rate to 0.350 µL/min over 1 min. Further ionization was performed by electrospray ionization technique (ESI) by NanoLockspray ionization source in a positive ion mode (capillary voltage at 3.80 kV and the source temperature at 100^{o}C). Quadrupole Time-of-Flight (Q-TOF) analyzer operated in a positive ion MS/MS mode was used for peptide mass detection. A MS/MS full-scan was performed for each sample with an acquisition m/z range of 0-1000 Da. Instrumental control and data analysis were executed using MassLynx software (Micromass U.K. Ltd., Wythenshawe, Manchester, U.K.). Peaks Viewer 4.5 (Bioinformatics Solutions Inc., Waterloo, ON, Canada) was used in combination with manual *de novo* sequencing to process the MS/MS data and to perform peptide

sequencing. The peptide sequences were identified from the respective monoisotopic mass.

Statistical analysis: All analysis were performed in triplicates and comparisons among the groups were carried out by one-way analysis of variance (ANOVA), grouped by Duncan's multiple range test and Tukey's studentized range test using Statistical Analysis System Software, SAS version 9.0 (SAS Institute, Cary, NC). Groups were considered to be significantly significant when $P \leq 0.05$ and results were reported as mean ± SEM.

Results and Discussion

Effects of solvent concentration and extraction time on the antioxidant activity: Based on the solubility, antioxidants can be grouped as hydrophilic antioxidants such as vitamin C and lipophilic compounds such as vitamin E and carotenoids.[29] Hydrophilic antioxidants circulate in the body, while lipophilic antioxidants can penetrate the lipoprotein cell membrane with increased bioavailability and serve as an *in vivo* free radicals chain breaking antioxidant.[31] It is difficult to determine the exact amount of lipophilic components in food, as the antioxidants components were of chemical diversity and were differentially localized. In eggs, antioxidant components are composed of proteins such as ovotransferrin and phosvitin, free amino acids, carotenoids, phospholipids, etc. Therefore, extraction of the lipophilic and hydrophilic fractions would help to determine the total antioxidant activity of the egg sample. To optimize the extraction conditions, effects of ethanol concentration and extraction time were studied. Table 1 illustrates the effect of solvent concentrations (20, 40, 60, 80 and 100 % ethanol for 1 h) on the scavenging property. Ethanol was used due to its nontoxic nature and environment friendly properties.[32-34] A gradual increase in the antioxidant activity was observed up to 80% while there was a decline at 100% ethanol. A similar trend was reported when ethanol was used beyond 70% for the antioxidant activity of extracts of Jerusalem artichoke.[35] The ethanol concentration influences the properties of the components by increasing the solvent to solid ratio and thereby increases the rate of diffusion of the compounds from the solid to the solvent.[36] The presence of diverse compounds with different polarity might have contributed to the altered antioxidant property of the hydrophilic fraction of egg yolk samples. Our study showed extraction at 80% ethanol concentration has the highest antioxidant activity (Table 1). Extraction time had significant effect on the antioxidant activity while the activity was not increased at prolonged extraction time (Table 2). Studies on ethanolic extracts of defatted borage (Borago officinalis L.) seeds in a meat model system showed neither short (15 min) nor long (105 min) extraction times are suitable for the optimum antioxidant activity and reported a maximum free radical scavenging activity at 62 min.[37] Our results showed the optimum time was 1 h (Table 2). The decrease in the antioxidant activity noticed after 1 h may be because of the oxidation of the antioxidative compounds due to the increased oxygen exposure over the.[38] As reported by Chew et al.[39], the time of extraction plays an important role in the reduction of energy as well as extraction process; hence it is well recommended to select least time with maximum extraction. In the study, 80% ethanol was chosen as the solvent concentration and 1 h as the extraction time.

Table 1: Effect of solvent concentrations on hydrophilic ORAC (H-ORAC) of fresh egg yolk.

Extraction solvent (Ethanol %)	**H-ORAC** (µmol TE/mg)	**Total ORAC** (µmol TE/mg)
Phosphate buffer (pH 7.5)	0.012 ± 0.04^{c}	0.031 ± 0.05^{d}
20	0.003 ± 0.03^{d}	0.040 ± 0.07^{c}
40	0.047 ± 0.04^{b}	0.075 ± 0.09^{b}
60	0.043 ± 0.03^{b}	0.071 ± 0.04^{b}
80	0.066 ± 0.02^{a}	0.094 ± 0.02^{a}
100	0.045 ± 0.04^{b}	0.073 ± 0.05^{b}

*The total antioxidant activity was calculated as the sum of H-ORAC and the lipophilic ORAC (L-ORAC) values. The statistical analysis of data was done using one-way analysis of variance (ANOVA) and was grouped using Duncan's multiple range test; different letters (a, b, c, d) denotes significant difference with the treatment groups ($P < 0.05$).

Table 2: Effect of extraction time on hydrophilic ORAC (H-ORAC) of fresh egg yolk.

Extraction time (h)	**H-ORAC** (µmol TE/mg)	**Total ORAC** (µmol TE/mg)
0.5	0.042 ± 0.04^{d}	0.070 ± 0.07^{d}
1	0.067 ± 0.03^{a}	0.095 ± 0.05^{a}
2	0.043 ± 0.04^{d}	0.071 ± 0.09^{d}
4	0.044 ± 0.03^{d}	0.072 ± 0.04^{d}
6	0.059 ± 0.02^{b}	0.087 ± 0.02^{b}
8	0.048 ± 0.04^{c}	0.076 ± 0.05^{c}
24	0.057 ± 0.04^{b}	0.085 ± 0.05^{b}

*The total antioxidant activity was calculated as the sum of H-ORAC and the lipophilic ORAC (L-ORAC) values. The statistical analysis of data was done using one-way analysis of variance (ANOVA) and was grouped using Duncan's multiple range test; different letters (a, b, c, d) denotes significant difference with the treatment groups ($P < 0.05$).

Effect of cooking and simulated digestion on the antioxidants: The antioxidant activity was measured by three different methods (ORAC, DPPH and ABTS) related with two different antioxidant

mechanisms: electron transfer (DPPH and ABTS) and radical stabilization (ORAC). Effect of cooking methods on the antioxidant activity of eggs was determined using hydrophilic and lipophilic ORAC assays (Table 3). The antioxidant activity of the lipophilic extract is 0.028 ± 0.05 µmol TE/mg. Among the egg white samples, the fresh samples showed higher antioxidant activity than the fried samples. But the fresh and boiled egg white samples did not show significant difference. The water-soluble amino acids and proteins, which possess the antioxidant activity by their metal chelating property[19], may contribute to the antioxidant activity of the fresh egg samples. Wu et al.[33] reported that cooking can alter the proteins, denature and degrade or reduce the antioxidant activity of compounds, especially the hydrophilic compounds. The digested egg white samples exhibited much higher ($P < 0.05$) antioxidant activity than the undigested ones (Table 3). This is due to the release of peptides and amino acids during digestion, which can act as primary antioxidants, or possess synergistic action.[25, 40] Fresh egg yolk showed higher antioxidant activity than fresh egg white (Table 3). The higher antioxidant activity of the egg yolk may be due to the presence of natural antioxidants present in the fresh sample. The egg yolk is a rich source of unsaturated fatty acids and iron [41]; in order to prevent the lipid peroxidation, there exist an antioxidant system within the egg yolk.[42] The presence of egg yolk components like phosvitin, egg yolk phospholipids such as sphingomyelin, lysophosphatidylcholine, phosphatidyl choline, phosphatidylethanolamine, carotenoids like lutein and zeaxanthin, free aromatic amino acids with reported antioxidant activity contributes to the overall radical scavenging activity of the egg yolk samples.[21, 22, 43, 44] It was also noticed that cooking reduced the antioxidant activity, which might be due to the destruction or degradation of the antioxidant components during cooking. Simulated gastrointestinal digestion led to significant increase in the antioxidant activity. The boiled egg yolk samples treated with pepsin followed by pancreatin showed higher antioxidant activity than the other treated groups. Our results were in alignment with previous reports that digestion release different compounds from the food matrix and improves the antioxidant activity of foods, even egg.[25, 40]

Antioxidant activity of the fresh whole egg samples was much lower than the fresh egg yolk (Table 3). This may be due to either an inefficient extraction of antioxidants from whole egg using one solvent, or the total antioxidant activity was masked by the interaction between proteins and carotenoids, similar to the masked effect was reported for the interaction between proteins and tea flavanoids.[45] Interestingly, our results showed that antioxidant activity of whole egg samples increased after cooking; this may be due to decreased protein and carotenoid interaction during cooking, leading to improved extraction of carotenoids from the samples. Possible synergistic antioxidant activity was not observed in fresh whole egg samples and the decrease observed in the homogenized whole egg samples might be due to the interaction between the components present in the egg white and egg yolk, thereby reducing the free radical scavenging property. Similarly, simulated gastrointestinal digestion of whole egg samples also increased the antioxidant activity in a similar trend as above.

DPPH radical scavenging activity and ABTS assay showed similar trends to that of ORAC (Tables 4 and 5). DPPH is a very strong chromogen and the presence of the antioxidants and an electron or hydrogen donor in a sample, result in the discoloration of the radical chromogen[4]; except in egg yolk samples, this activity was not reduced by cooking and was significantly increased upon digestion of cooked eggs. ABTS assays showed slight difference in the activity among fresh and cooked egg white samples, as well as whole egg samples, but not in egg yolk samples**.** But it was noticed that boiled samples treated with pepsin and pancreatin showed significantly higher antioxidant activity than the fried pepsin and pancreatin treated samples. Among the whole egg samples, boiled samples showed no different from the fried samples (Table 4). The present study showed the presence of antioxidants in eggs, and the antioxidant activity increased upon simulated digestion. All the assays showed an increase in antioxidant activity subjected to digestion; these findings coincided with other observations on the increased antioxidant activity of peptides derived from egg yolk [27, 46, 47] and egg white.[15] Thus, this study shows the potential role of egg in

Table 3: Total antioxidant (lipophilic and hydrophilic) activity of the egg samples based on ORAC assay.

Samples	**H-ORAC** (µmol TE/mg)	**L-ORAC** (µmol TE/mg)	**Total ORAC** (µmol TE/mg)
Egg white			
Fresh - No enzyme	0.058 ± 0.32^{i}	-	0.058 ± 0.32^{l}
Boiled - No enzyme	0.056 ± 0.12$^{i, j}$	-	0.056 ± 0.12^{l}

Pepsin	0.129 ± 0.01[c,d]	-	0.129 ± 0.01[g]
Pepsin+ Pancreatin	0.197 ± 0.10[a]	-	0.197 ± 0.10[a]
Fried - No enzyme	0.052 ± 0.04[j]	-	0.052 ± 0.04[m]
Pepsin	0.115 ± 0.03[e]	-	0.115 ± 0.03[h]
Pepsin+Pancreatin	0.151 ± 0.24[b]	-	0.151 ± 0.24[e]
Egg yolk			
Fresh - No enzyme	0.065 ± 0.04[h]	0.027 ± 0.05[e]	0.092 ± 0.08[i]
Boiled - No enzyme	0.059 ± 0.14[i]	0.020 ± 0.07[f]	0.079 ± 0.12[j]
Pepsin	0.117 ± 0.05 [e]	0.030 ± 0.04[d]	0.147 ± 0.09[f]
Pepsin+Pancreatin	0.120 ± 0.09[e]	0.059 ± 0.12[a]	0.179 ± 0.08[c]
Fried - No enzyme	0.055 ± 0.02[i, j]	0.021 ± 0.09[f]	0.076 ± 0.02[j]
Pepsin	0.102 ± 0.13[g]	0.031 ± 0.04[d]	0.133 ± 0.18[f]
Pepsin+ Pancreatin	0.105 ± 0.07[g]	0.061 ± 0.12[a]	0.166 ± 0.04[d]
Whole egg			
Fresh - No enzyme	0.038 ± 0.05[k]	0.026 ± 0.12[e]	0.064 ± 0.07[k]
Boiled - No enzyme	0.055 ± 0.11[i,j]	0.023 ± 0.07[f]	0.078 ± 0.04[j]
Pepsin	0.142 ± 0.04[c]	0.022 ± 0.12[f]	0.164 ± 0.09[d]
Pepsin+Pancreatin	0.129 ± 0.07[c, d]	0.052 ±0.03[b]	0.181 ± 0.13[b]
Fried - No enzyme	0.052 ± 0.04[j]	0.025 ± 0.09[e]	0.077 ± 0.02[j]
Pepsin	0.111 ± 0.05[f]	0.018 ± 0.03[g]	0.129 ± 0.06[g]
Pepsin+ Pancreatin	0.120 ± 0.10[e]	0.042 ± 0.02[c]	0.164 ± 0.13[d]

*The statistical analysis of data was done using one-way analysis of variance (ANOVA) and was grouped using Tukey's studentized range test; alphabets denotes significant difference with the treatment groups (P <0.05). Data represent mean ± SEM; n=3

Table 4: Total antioxidant (lipophilic and hydrophilic) activity of the egg samples based on DPPH assay.

Samples	**H-DPPH** (µmol TE/mg)	**L-DPPH** (µmol TE/mg)	**Total DPPH** (µmol TE/mg)
Egg white			
Fresh - No enzyme	0.019 ± 0.09[j]	-	0.019 ± 0.09[i]
Boiled - No enzyme	0.023 ± 0.04[i]	-	0.023 ± 0.04[g]
Pepsin	0.045 ± 0.12[e]	-	0.045 ± 0.12[e]
Pepsin+ Pancreatin	0.058 ± 0.09[d]	-	0.058 ± 0.09[d]
Fried - No enzyme	0.026 ± 0.19[h]	-	0.026 ± 0.19[f, g]
Pepsin	0.056 ± 0.03[d]	-	0.056 ± 0.03[d]
Pepsin+ Pancreatin	0.053 ±0.07[d,e]	-	0.053 ± 0.07[d]
Egg yolk			
Fresh - No enzyme	0.017±0.02[k]	0.004 ±0.002[c]	0.021 ± 0.02[h]
Boiled - No enzyme	0.017±0.01[k]	0.001±0.001[d]	0.018 ± 0.07[i]
Pepsin	0.035±0.02[f]	0.010±0.021[b]	0.045 ± 0.04[e]
Pepsin+Pancreatin	0.046±0.02[e]	0.011±.001[a]	0.057 ± 0.02[d]
Fried - No enzyme	0.019±0.02[j]	0.001±.004[d]	0.020 ± 0.09[h]
Pepsin	0.020±0.02[i]	0.003±0.002[d]	0.023 ± 0.14[g]
Pepsin+Pancreatin	0.028±0.02[g]	0.002±0.001[e]	0.030 ± 0.22[f]
Whole egg			
Fresh - No enzyme	0.016±0.02[k]	0.002±0.01[e]	0.018 ± 0.05[i]
Boiled - No enzyme	0.025±0.02[h]	0.002±0.003[e]	0.027 ± 0.17[f, g]
Pepsin	0.068±0.02[c]	0.001±0.011[d]	0.069 ± 0.28[c]
Pepsin+ Pancreatin	0.077±0.02[a]	0.001±0.009[d]	0.078 ±0.04[a]
Fried - No enzyme	0.023±0.02[i]	0.002±0.001[e]	0.025 ± 0.07[f, g]
Pepsin	0.052±0.02[d,e]	0.004±0.002[c]	0.056 ± 0.33[d]
Pepsin+Pancreatin	0.069±0.02[b]	0.004±0.01[c]	0.073 ± 0.21[b]

*The statistical analysis of data was done using one-way analysis of variance (ANOVA) and was grouped using Tukey's studentized range test; superscripts of alphabets denotes significant difference with the treatment groups (p <0.05). Data represent mean ± SEM; n=3.

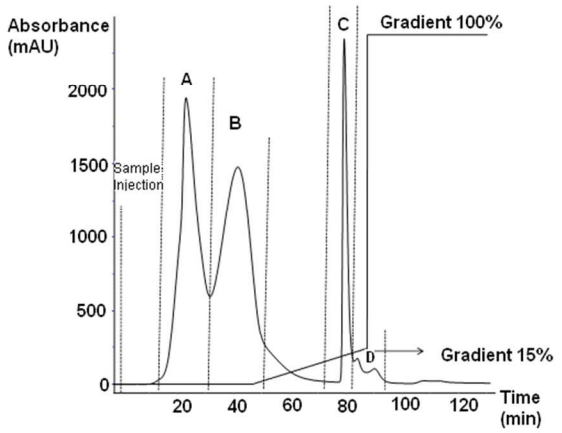


Figure 1. Cation exchange chromatogram of boiled egg white hydrolysate using HiPreP 16/10 SP FF cation exchange column as described in materials and methods.

the diet as a source of antioxidants that might contribute to the prospective benefits of egg consumption.

Fractionation of antioxidant peptides from boiled egg white hydrolysate: Boiled egg white subjected to pepsin and pancreatin enzymatic hydrolysis possessed the higher antioxidant activity than that of egg yolk; hence the boiled egg white hydrolysate was further purified and characterized for the study of antioxidants. Cation exchange chromatography of boiled egg white hydrolysate resulted in three major peaks (A, B and C) and one minor peak (D) as shown in Fig. 1.

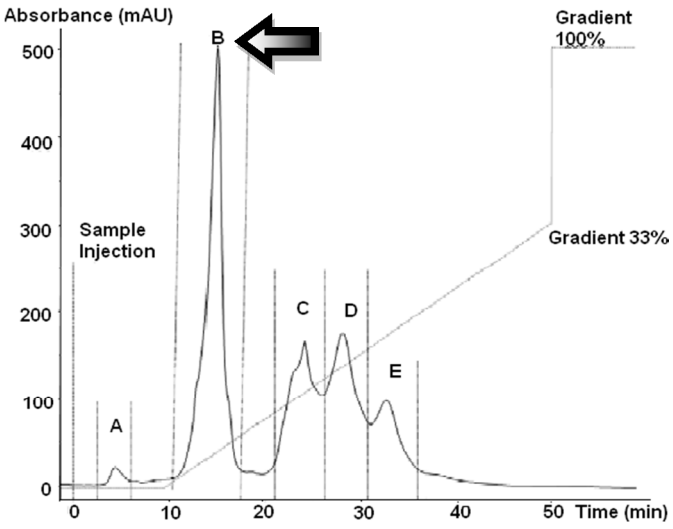


Figure 2. Anion exchange chromatogram of Fraction A, which exhibited the most potent antioxidant activity using HiPrep Q FF 16/10 anion exchange column as described in 3.2.5.

The antioxidant activity was determined for all the fractions using ORAC, ABTS and DPPH assays (*data not shown*). The fraction A showed the most potent antioxidant activity was then subjected to anion exchange chromatography (Fig. 2). Five fractions were collected and the antioxidant activity was determined (*data not shown*). The most potent fraction B was then subjected to further purification using an Xbridge C18 RP-HPLC column. A total of 19 fractions were collected (Fig. 3) and the antioxidant activity of each fraction was analyzed (*data not shown*); fractions 1, 5, 8 and 14, which exhibited the highest antioxidant activity were used for further analysis by LC-MS/MS. Fraction 12 showed the highest peptide concentration was also subjected for characterization.

Identification of peptide sequences: MS spectrums of each fraction and one representative peptide MS/MS interpretation from each fraction is shown in Fig 4. Peptides having intensity above the cutoff of 40% were sequenced using Peaks Viewer 4.5 (Bioinformatics Solutions Inc., Waterloo, ON, Canada) in combination with manual *de novo* sequencing to process the MS/MS results (Table 6). A total of 63 peptides derived from boiled egg white were identified: 10 peptides from F1, 11 from F5, 13 from F8, 16 from F12, and 13 from F14 with amino acid residues ranging from 3 to 10.

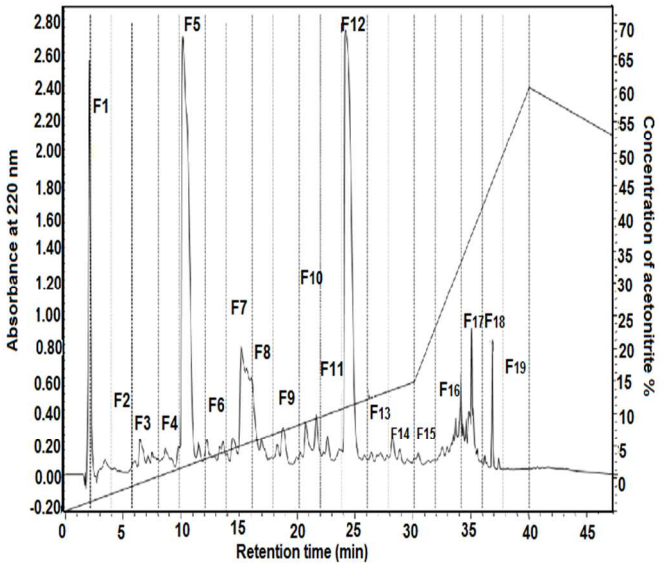


Figure 3. RP-HPLC chromatogram of fraction B in Figure 3.3 by Xbridge C18 column (10 mm x 150 mm, 0.5 M) under linear gradient condition of 100% solvent A (0.1%TFA in water) to 40% solvent B (0.1% TFA in acetonitrile) over 40 min at a flow rate of 5 mL/m

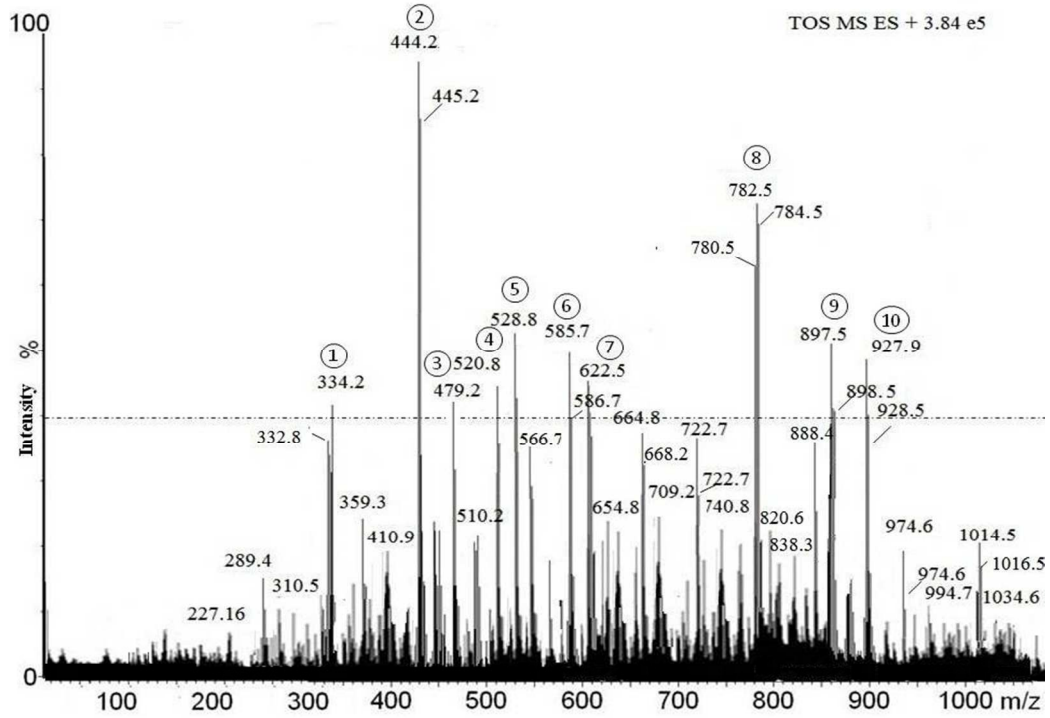
Table 5: Total antioxidant (lipophilic and hydrophilic) activity of the egg samples based on ABTS assays.

Samples	H-ABTS (µmol TE/ mg)	L-ABTS (µmol TE/mg)	Total ABTS (µmol TE/mg)
Egg white			
Fresh - No enzyme	0.049 ± 0.05^{i}	-	0.049 ± 0.05^{l,m}
Boiled - No enzyme	0.051 ± 0.11^{i}	-	0.051 ±0.11$^{k, l}$
Pepsin	0.103 ± 0.07^{c}	-	0.103 ± 0.07^{f}
Pepsin+Pancreatin	0.116 ± 0.20^{b}	-	0.116 ±0.20^{c}
Fried - No enzyme	0.045 ± 0.04^{j}	-	0.045 ±0.04^{m}
Pepsin	0.086 ± 0.15^{d}	-	0.086 ±0.15^{h}
Pepsin+Pancreatin	0.126 ± 0.03^{a}	-	0.126 ±0.03^{a}
Egg yolk			
Fresh - No enzyme	0.050 ± 0.09^{i}	0.034 ± 0.01$^{c, d}$	0.084 ±0.11^{h}
Boiled - No enzyme	0.018 ± 0.02^{n}	0.029 ± 0.07^{e}	0.047 ±0.08^{m}
Pepsin	0.061 ± 0.06^{h}	0.046 ± 0.01^{a}	0.107 ±0.02^{e}
Fried - No enzyme	0.039 ± 0.02^{l}	0.028 ± 0.07^{e}	0.067 ±0.09^{j}
Pepsin	0.067 ± 0.10^{g}	0.032 ± 0.11^{d}	0.099 ±0.13^{g}
Whole egg			
Fresh - No enzyme	0.044 ± 0.02^{k}	0.035 ± 0.04^{c}	0.079 ±0.06^{k}
Boiled - No enzyme	0.044 ± 0.02^{k}	0.040 ± 0.04^{b}	0.084 ±0.06^{h}
Pepsin	0.069 ± 0.05^{f,g}	0.028 ± 0.02^{e}	0.097 ±0.07^{g}
Pepsin+Pancreatin	0.076 ± 0.09^{e}	0.045 ±0.04^{a}	0.121 ±0.14^{b}
Fried - No enzyme	0.039 ± 0.02^{l}	0.029 ± 0.03^{e}	0.068 ±0.05^{j}
Pepsin	0.070 ± 0.12^{f}	0.036 ± 0.03^{c}	0.106 ±0.32^{e}
Pepsin+Pancreatin	0.071 ± 0.06^{f}	0.040 ± 0.01^{b}	0.111 ±0.07^{d}

*The statistical analysis of data was done using one-way analysis of variance (ANOVA) and was grouped using Tukey's studentized range test; superscripts of alphabets denotes significant difference with the treatment groups ($p < 0.05$). Data represent mean ± SEM; n=3.

Ovalbumin, contributing to 54-58% (w/w) of the total egg white protein, contains 386 amino acids sequences with a molecular weight of 45 kDa. [48-50] 18 peptides identified from the pepsin and pancreatin hydrolysate of boiled egg white were derived from ovalbumin. Several studies revealed the presence of antihypertensive peptides like RADHPFL[51], YAEERYPIL[15], and IVF present in the egg white hydrolysates.[52] YAEERYPIL was also characterized as a potent radical scavenging peptide.[15] Ovotransferrin, the second major egg white protein (12-14 %, w/w), consists of 686 amino acid residues with a molecular mass of 78 kDa. This is a disulfide bond rich single chain glycoprotein that has been reported to have involvement in the redox linked signals and response to free radicals and specifically attacks superoxide radicals.[17, 49, 53] A total of 19 antioxidative peptides identified from boiled egg white hydrolysate were derived from ovotransferrin. LGFEYY (residues 339-344) characterized from the study was also reported from our previous study as a potent antioxidant peptide.[54] Antioxidant peptides were also released from lyzozyme (5 peptides), ovostatin (6), ovomucoid (1), ovomucin α (7) and β (4) subunits and flavoprotein (3). The lyzozyme has a role in protecting against the oxidative damage in the body.[55] Ovomucin was reported for its immunomodulatory property [56]; it is interesting to note a total of 11 peptides were characterized from ovomucin in the study. Ovostatin was reported as an antimicrobial protein[57]; our study showed its derived peptides also possess antioxidant activity. It was recently reported that peptide containing Pro (P), Asp (D), Tyr (Y), Trp (W) or His (H) tends to show greater antioxidant activity.[24, 58] Pyrrolidine ring present in the proline has remarkably low ionization potential and forms charge transfer complex with $^1O_2$ while proline forms stable radicals with $^{\bullet}$OH [59]; thus proline acts as a scavenger of $^1O_2$ and $^{\bullet}$OH, in addition to its reaction to H_2O_2 induced [60-62]; 27 peptides identified in the study contain proline (Table 6). The presence of indole group in Trp (W) and phenol group in Tyr serves as potent hydrogen donors that could help in converting the reactive oxygen species to more stable and less active indoyl and phenoxyl radicals.[24, 63] The presence of Trp was found in peptides from ovotransferrin (RIQWCAVGKD, SAGWN), ovalbumin (WTSSN) and ovostatin (GWIESPS). Tyr, another amino acid with antioxidant property was present in peptides from ovalbumin (2), ovotransferrin (3), ovmucin (2) and lyzozyme (2). Recent quantitative structure and activity relationships of antioxidant peptides indicated that a peptide with a hydrophobic amino acid at N-terminus, a basic amino acid residue at C-terminus, and a hydrophilic amino acid residue next to C terminus shows greater antioxidant activity.[64] Hydrophobic amino

A (a) Fraction 1



A (b)

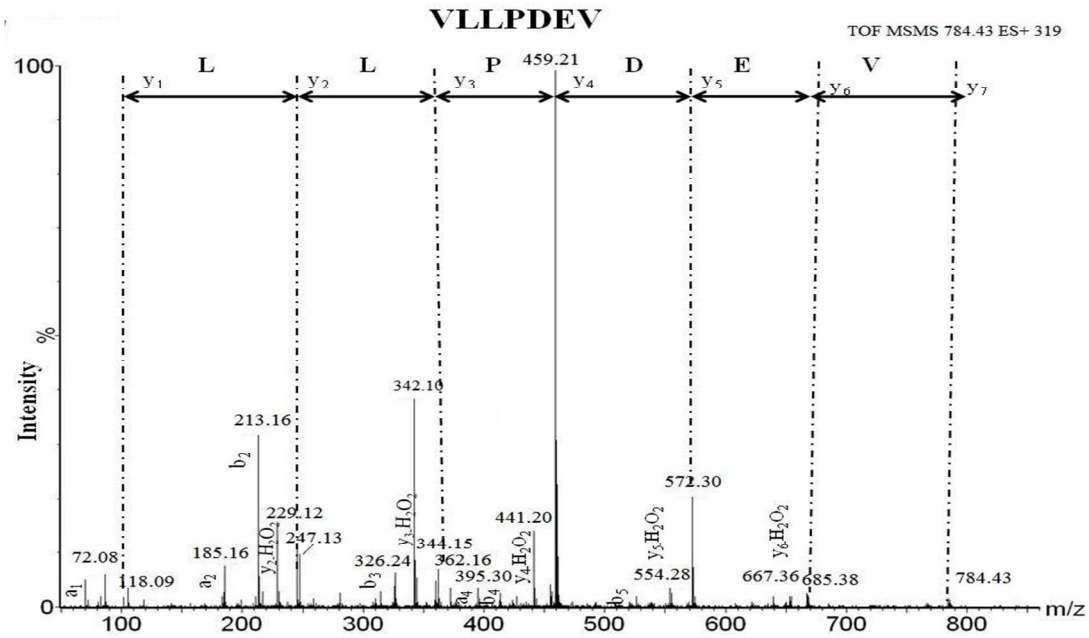


Figure 4. LC-MS spectra of fractions from RP-HPLC. The dashed line represents the cutoff ion intensity (40 %) of selected parent ions in the peptide sequencing. One candidate peptide was shown as de novo sequencing by using their MS/MS spectra by monoisotopic mass of the amino acids. A(a) Fraction 1 (1-10 parent ions); A (b) Interpretation of LC-MS/MS spectrum of the ion *m/z* 784.45, derived from ovalbumin peptide VLLPDEV.

acid residues such as Val or Leu at the N terminus were also reported to increase the antioxidant activity.[24, 64] The ovalbumin derived peptides LQPSSVD and VLQPSSVD, and flavoprotein-derived peptide VAQ and VPN, contain Val or Leu at their N termini, suggestive of increased antioxidative property.[24] Among the total 10 peptides identified from fraction 1 (F1), 6 peptides (VPGAT, LHPI, LVELI, VKYNV, VLLPDEV, and LVLLPDEV) possess Val or Leu as the N terminus. The imidazole ring in His contributes to the antioxidant activity as a proton donor and a metal chelator.[24, 64, 65] It was reported previously that the removal of the histidine from the C terminus could decrease the antioxidant activity of the peptides.[66] Tsuge *et al.*[67] reported 3 peptides (AHK, VHH, and VHHANQN) from egg proteins containing His and Val residues with strong antioxidant property. In this study, histidine containing peptides, AAHAV, LAEVPTH and VAAH from ovotransferrin, and AVHAAH from ovalbumin, were identified. Amino acid residues such as Ile, Phe, Ala and Lys at the N terminus also increase antioxidant activity.[68] 17 peptides identified in the study contain one of these amino acid residues, which might contribute to the antioxidant activity of the peptides.

Table 6. Peptide sequences identified by LC-MS/MS from RP-HPLC fractions (see Fig 3).

Molecular ion (m/z) selected for MS/MS Charge)	Sequence	Source Fragment (f)
Fraction 1		
1) 334.2 (1)	SGGI	Ovotransferrin f (524 -527)
2) 444.22 (1)	VPGAT	Ovotransferrin f (180-184)
3) 479.44 (1)	LHPI	Ovostatin f (608-611)
4) 520.75 (2)	YAEERYPIL	Ovalbumin f (107-115)
5) 528.8 (2)	RIQWCAVGKD	Ovotransferrin f (363 -372)
6) 585.66 (1)	LVELI	Ovomucin α unit f (1457-1461)
7) 622.46 (1)	VKYNV	Ovomucin β unit f (933-937)
8) 784.45 (1)	VLLPDEV	Ovalbumin f (244 -250)
9) 897.5 (1)	LVLLPDEV	Ovalbumin f (243-250)
10) 927.89(1)	RNAPYSGY	Ovotransferrin f (203 -210)
Fraction 5		
1) 303.26 (1)	AGVG	Lysozyme f (1 5 -178)
2) 349.18 (1)	ACR	Ovomucin β unit f (345-347)
3) 371.2 (1)	AGHS	Ovos atin f (1099-1102)
4) 428.48 (1)	PGKK	Ovotransferrin f (307-310)
5) 533.52 (1)	SAGWN	Ovotransferrin f(241-245)
6) 589.41 (1)	ASNGIQ	Ovomucin β unit (97- 102)
7) 633.21 (1)	QTAADQ	Ovalbumi f (135-140)
8) 719.3 (1)	KVEQGAS	Ovomucoid f (136-142)
9) 755.58 (1)	YCGVRAS	L sozyme f (54-60)
10) 771.64 (1)	RAAAARGV	Flavoprotein f (3-10)
11) 919.72 (1)	IESGSVEQA	Ovotransferrin f (162-170)
Fraction 8		
1) 346.18 (2)	LGAKDST	Ovalbumin f (44-50)
2) 465.25 (1)	CQGGT	Lysozyme f (24-28)
3) 468.29 (1)	AAHAV	Ovotransferrin f (267-271)
4) 481.26 (1)	FDVT	Ovostatin f (221-224)
5) 553.24(1)	ASGTMS	Ovalbumin f (236-241)
6) 565.2 (1)	TGEIK	Ovostain f (496-500)
7) 584.2 (1)	VCGLVP	Ovotransferrin f (423-428)
8) 594.36 (1)	WTSSN	Ovalbumin f (268-272)
9) 691.36 (1)	LGAKDST	Ovalbumin f (44-50)
10) 766.4 (1)	LAEVPTH	Ovotransferrin f (605-611)
11) 791.2 (1)	LGFEYY	Ovotransferrin f (339-344)
12) 815.56 (1)	QESKPVQ	Ovalbumin f (204-210)
13) 826.65 (1)	DVFSSSAN	Ovalbumin f (305-312)
Fraction 12		

1)	317.14 (1)	VAQ	Flavoprotein f (64-66)
2)	329.2 (1)	VPN	Flavoprotein f (258-260)
3)	345.2 (1)	GAVV	Ovomucin α unit f (882-1885)
4)	371.22 (1)	PAGT	Ovomucin α unit f (350-353)
5)	397.1 (1)	VAAH	Ovotransferrin f (267-269)
6)	432.07 (1)	LKDG	Ovotransferrin f (207-210)
7)	445.19 (1)	PTDI	Ovomucin α unit f (663-665)
8)	488.74 (2)	TVNDLQGKTS	Ovotransferrin f (124-132)
9)	522.5 (2)	YNAGV	Lysozyme f (173-177)
10)	524.26 (2)	TVNDLQGK	Ovotransferrin f (124-131)
11)	569.14 (1)	VVVDP	Ovotransferrin f (613-617)
12)	590.73 (1)	AGLAPY	Ovotransferrin f (86-91)
13)	597.2 (1)	TKSDF	Ovotransferrin f (297-301)
14)	642.5 (1)	LVEPEG	Ovostatin f (886-888)
15)	798.4 (1)	QITKPND	Ovalbumin f (90-96)
16)	977.47 (1)	TVNDLQGKT	Ovotransferrin f(124-132)
Fraction 14			
1)	471.38 (1)	KPGAV	Ovomucin α unit f (1880-1884)
2)	518.75(2)	ITKPNDVYS	Ovalbumin f (91-99)
3)	532.16 (1)	KGGISA	Lysozyme f (167-172)
4)	542.7 (1)	ATALAP	Ovomucin α unit f (1362-1367)
5)	579.27 (1)	PFASGT	Ovalbumin f (234-239)
6)	604.52 (1)	AVHAAH	Ovalbumin f (317-322)
7)	622.66 (1)	YAPGDT	Ovomucin β unit f (336-341)
8)	688.6 (1)	GWIESPS	Ovostain f (423-428)
9)	745.59 (1)	LQPSSVD	Ovalbumin f (162-168)
10)	765.38 (1)	ETTQGMS	Ovomucin α unit f (966-972)
11)	845.4 (1)	VLQPSSVD	Ovalbumin f (161-168)
12)	913.9 (1)	QITKPNDV	Ovalbumin f (90-97)
13)	1036.49 (1)	ITKPNDVYS	Ovalbumin f (91-99)

Conclusions

Antioxidants are present both in egg white and egg yolk; fresh egg yolk shows higher antioxidant activity than the fresh egg white and the whole egg samples. The antioxidant activity of the egg samples tested by different assays resulted in similar trends on the effect of cooking and simulated gastrointestinal digestion. Cooking reduced antioxidant activity of egg yolk more than egg white and whole egg. Simulated gastrointestinal digestion increased significantly the antioxidant activity of all egg samples, which indicated the contribution of released peptides and amino acids. A total of 63 peptides were identified, mainly from ovalbumin, ovotransferrin, ovomucin, lysozyme, and ovostatin. Our previous study has shown the presence of antioxidative aromatic amino acids in egg yolk; results from the present study implied that gastrointestinal digestion of egg white proteins could further enhance the antioxidant activity of egg by releasing a number of antioxidant peptides from egg proteins. Further research on the antioxidant activity of each peptide *in vivo* will help to understand the most potent peptide from the boiled egg white hydrolysates.

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Notes and references

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There is no conflict of interest between authors.

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