Tandem mass tag-based quantitative proteomics analysis and gelling properties in egg albumen of laying hens feeding tea polyphenols

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ABSTRACT The tea polyphenol (TP) can improve the egg albumen quality in laying hens; however, our understanding of the molecular mechanisms and proteomic changes in the egg albumen remains limited. A total of 720 layers (35-wk-old) were allocated into 5 treatments with TP and were added at 0 (control), 200 (TP200), 400 (TP400), 600 (TP600), and 800 (TP800) mg/kg. It showed that 400 mg/kg TP increases albumen height and Haugh unit (quadratic effect, $P < 0.01$), while 400 mg/kg TP decreases gel strength, hardness, gumminess, and chewiness value in a quadratic manner ($P = 0.01$). Eggs from TP400-fed layers had highest reducing power and oxygen radical absorbance capacity, and lowest albumen malondialdehyde content (quadratic effect, $P < 0.05$). Through Tandem Mass Tag-based quantitative proteomics analysis, 258 proteins were identified and 31 differentially accumulated proteins in egg white affected by 400 mg/kg TP compared to control group, with 19 proteins upregulated and 12 proteins downregulated. A total of 11 binding proteins (A0A1D5PZE3, F1NTQ2, Q7SX63, F1NRV5, P24802, A0A1L1RM02, E1BTX1, A0A1L1RMF4, A0A1D5P1N3, A0A1L1RML6, A0A1L1RQF3), 9 immune response proteins (P10184, R4GI90, P01875, Q6IV20, Q64EU6, P02701, P08110, P0CB50, A0A1D5PQ63), and 3 cell redox homeostasis proteins (P0CB50, P20136, Q8JG64) were changed in albumen of laying hens fed TP400. The differentially expressed proteins mainly involved in pyruvate metabolism, cysteine and methionine metabolism, glutathione metabolism, glycolysis, and protein processing in endoplasmic reticulum pathway. The result gathered in this study suggested that the improving mechanism of TP on albumen quality may act through regulating binding mediation, immune function, and antioxidant activity-related proteins.

Key words: egg white, tea polyphenols, antioxidative capacity, quantitative proteomic

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INTRODUCTION

The chicken eggs are known for their nutritional values, and contain a variety of biological activities such as anti-bacterial, anti-fungal, anti-viral, anti-inflammatory, anti-hypertensive, as well as antioxidant properties (Kovacs-Nolan et al., 2005). A fresh and high-quality egg should have a firm and gelatinous albumen for anchoring the yolk and maintaining antimicrobial effect. Albumen quality is measured by Haugh unit (**HU**), expressed as a function of egg weight and the albumen height of a broken egg. On the other hand, albumen has many functional properties, such as gel formation and water-holding and whipping capacity (Baron et al., 2003).

Tea polyphenol (**TP**) is a natural antioxidant of typical flavonoids, which can scavenge active oxygen free radicals produced in many systems and protect cells from damage (Frei and Higdon, 2003; Ariana et al., 2011). It has been observed that supplementing laying hens' diets with green tea extract improved egg production, feed efficiency, and egg white quality of eggs during the late laying period (Afzal et al., 2015; Wang et al., 2017, 2018). However, the majority of the studies related to TP or epigallocatechin gallate (**EGCG**) mainly concentrate on the changes in lipid metabolism and antioxidant functions in human beings or mice model, whereas the effect of TP on the antioxidant capacity of eggs and its mechanism is rather scarce.

Therefore, the objective of this study is to investigate the response of hens to dietary TP supplementation by evaluating the egg antioxidant capacity and TMT-based quantitative proteomics. These results would contribute to further deciphering of molecular mechanisms underlying the response of layers to TP.

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MATERIALS AND METHODS

Preparation of TP

TP was purchased from Red Star Pharmaceutical (Co. Ltd. Anhui, China), with 98.6% purity, which contains 66.3% of EGCG, 16.5% of epigallocatechin, 7.8% of epicatechin-3-gallate, 5.7% of epicatechin (**EC**), and 0.4% of caffeine.

Birds, Diets, and Management

The experimental protocol used in the study was approved by the Animal Care and Use Committee of Sichuan Agricultural University. A total of 720 35-wk-old Lohmman laying hens were chosen and randomly allocated into 5 treatments (6 replicates/treatment, 24 birds/replicate). All diets were formulated according to the recommendations of published NRC (1994) and shown in Table 1. Layers were fed diets included 0 (control), 200 (**TP200**), 400 (**TP400**), 600 (**TP600**), and 800 (**TP800**) mg/kg of TP for 8 wk. Layers were kept in a windowless room at the temperature of 20°C, humility of 60%, and light of 16 h. Feed and water were given ad libitum consumption throughout the whole experimental period.

Table 1. Ingredients and chemical composition of the basal diet (g/kg, as fed basis).

Ingredients	Content, g/kg
Corn	590.64
Wheat bran	38.67
Soybean oil	15.00
Soybean meal, 43% CP	152.36
Corn gluten meal	50.00
Corn DDGS	50.00
$CaCO_3$	86.03
$CaHPO_4$	9.41
NaCl	2.50
$NaHCO_3$	1.00
L-Lysine	1.62
DL-Methionine	0.12
Choline chloride	1.00
Vitamin premix[1]	0.15
Mineral premix[2]	1.50
Total	1,000.00
Analyzed nutrient levels	
AME (kcal/kg)[3]	2,690
Crude protein, %	16.00
Ether extract, %	4.39
Crude fiber, %	2.69
Calcium, %	3.70
Available phosphorus, %	0.36
Lysine, %	0.65
Methionine, %	0.33
Methionine + Cysteine, %	0.23

[1]Supplied per kilogram of diet: vitamin A, 12,000 IU; vitamin D_3, 3,000 IU; vitamin E 7.5 IU; vitamin K_2, 1.5 mg; vitamin B_1, 0.6 mg; vitamin B_2, 4.8 mg; vitamin B_6, 1.8 mg; vitamin B_{12}, 0.009 mg; pantothenic acid, 7.5 mg; folic acid, 0.15 mg/kg; niacin, 10.5 mg.

[2]Supplied per kilogram of diet: 60 mg Mn (as $MnSO_4 \cdot H_2O$); 80 mg Zn (as $ZnSO_4 \cdot 7H_2O$); 8 mg Cu (as $CuSO_4 \cdot 5H_2O$); 60 mg Fe (as $FeSO_4 \cdot 7H_2O$); 0.35 mg I (as KI); 0.3 mg Se (as $Na_2SeO_3 \cdot 5H_2O$).

[3]Calculated by NRC (1994).

Egg Quality

Thirty eggs were randomly collected from each treatment (5 eggs per replicate) at the end of the experiment. Albumen height and HU were measured by an automatic egg quality analysis instrument (EMT-5200, Robotmation Co., Ltd., Tokyo, Japan). Then the thick albumen and thin albumen were separated by 40 mesh stainless steel screen, and weighed.

Albumen Gelling Properties

At the end of the experiment, 24 eggs (4 egg per replicate) were randomly collected from each treatment for the gelling properties determination according to the previous method (Houska et al., 2004). Briefly, surface of the egg was wiped with alcohol cotton, albumen and egg yolk were separated, albumen was kept in 100 mL beaker, and mixed with a magnetic stirrer. Then 15 mL albumen was transferred into the 25 mL beakers, sealed with plastic wrap, placed in 80°C water bath to heat for 45 min. The heated gel and the beaker were quickly removed, cooled to room temperature, and placed in a refrigerator at 4°C for 24 h. The gel was measured by TA-XTplus2 with the TPA mode. The pre-test speed was 2.0 mm/s, the test speed was 5 mm/s, the post-test speed was 5 mm/s, and the probe was P/0.5 cylindrical. Hardness was defined by peak force (g) during the first compression cycle. Springiness was defined as a ratio of the time recorded between the start of the second area and the second probe reversal to the time recorded between the start of the first area and the first probe reversal. Cohesiveness was calculated as the ratio of the area under the second curve to the area under the first curve (dimensionless).

Evaluation on the Antioxidant Substances and Antioxidant Capacities of Egg White

Egg samples (6 replicates/treatment, 4 eggs/replicate) were also collected at the end of trial, egg whites were separate carefully and freeze-dried to measure chemical composition and antioxidant capacity.

Analysis of Amino Acids in Egg White Freeze-dried egg white powders were mixed into 88% formic acid and incubated overnight. The amino acid profiles were then determined by an L-8900 automatic Amino Acid Analyzer (Hitachi Ltd., Tokyo, Japan). Each egg yolk and egg whites were analyzed in triplicates.

Oxygen Radical Absorbance Capacity and Reducing Power Assay The oxygen radical absorbance capacity (**ORAC**) assay was followed by the description of previous reports (Ou et al., 2001; Liu et al., 2015). The reaction mode pipetted and transferred the sample (20 μL), phosphate buffer (5 μL, 75 mM, pH 7.4), and main reagent (365 μL FL, 48 nM) into the main reagent wells of their respective cuvette rotor positions. After shaking for 30 s, the microplate was immediately placed

into the Synergy microplate reader (Bio-Tek Instruments, Inc., Winooski, VT), and recorded every minute for 100 min. The fluorescence was set at 485 nm with a tolerance of ±20 nm as the excitation wavelength, and 528 nm ± 20 nm as the emission wavelength.

The reducing power (**RP**) of egg white samples were measured according to the method as the previous study presented (Ren et al., 2014). Briefly, sample (1 mg/mL) was added with 2.5 mL phosphate buffer saline (0.2 M) and $K_3[Fe(CN)_6]$ solution (1%, w/v). After the mixture incubated in water bath (50°C) for 20 min, the TCA solution (10%, w/v) was added and 2.5 mL of supernatant was collected after centrifugation (10 min, 3,000× *g*). The mixture was then measured for absorbance at 700 nm wavelength after 10 min at room temperature.

Determination of Total Antioxidant Capacity and Malondialdehyde in Egg The total antioxidant capacity (**T-AOC**) and malondialdehyde (**MDA**) content in egg white and yolk were measured by means of commercial kits (Nanjing Jiancheng Biotechnology, Nanjing, China).

Egg Albumen Protein Extraction and Peptides Preparation

Eggs were collected from control group and 400 mg/kg TP group (6 replicates/treatment, 2 eggs/replicate), and the egg whites were carefully separated from yolk and gently homogenized with a magnetic stirrer for 15 min to reduce the viscosity and stored at –80°C. The sample was grinded by liquid nitrogen into powder and then transferred to a 5-mL centrifuge tube. After that, 4 volumes of lysis buffer (8 M urea, 1% Protease Inhibitor Cocktail) was added to the powder, followed by sonication 3 times on ice using a high intensity ultrasonic processor (Scientz). The remaining debris was removed by centrifugation at 12,000 *g* under 4°C for 10 min. Finally, the supernatant was collected and the protein concentration was determined with BCA kit according to the manufacturer's instructions.

TMT Labeling and Proteome Analysis

For digestion, protein solutions were reduced with 5 mM dithiothreitol for 30 min at 56°C and alkylated with 11 mM iodoacetamide for 15 min at room temperature in darkness followed by the dilution to urea concentration <2 M by adding 100 mM triethylammonium bicarbonate. Subsequently, proteins were digested successively with trypsin (1:50 trypsin/protein) overnight and with trypsin (1:100 trypsin/protein) for 4 h at 37°C. The tryptic peptides were fractionated into fractions by high pH reverse-phase HPLC using Agilent 300Extend C18 column (5 μm particles, 4.6 mm ID, 250 mm length). Peptide mixture was labeled with a TMT isobaric tagging reagent according to the manufacturer's instructions (Applied Biocystems, CA, USA).

Bioinformation Analysis

To better understand the annotation and distribution of protein functions, we used the Blast2GO program to obtain Gene Ontology (**GO**) annotations. GO has 3 ontologies which can describe molecular function, cellular component, and biological process, respectively. The Kyoto Encyclopedia of Genes and Genomes (**KEGG**) pathway online service tools KAAS were used to annotated protein's KEGG database description.

Statistical Analysis

All data were analyzed using one-way ANOVA and linear regression analysis followed by a Duncan's multiple comparison test that was used to separate different means among treatments (SPSS 19.0). The data were expressed as the means ± standard deviation. Data were assumed to be statistically significant when $P \leq 0.05$.

RESULTS

Egg Quality

TP supplementation increased the albumen height and HU in a quadratic manner, with eggs from TP400 and TP600 groups having higher albumen height and TP400 having higher HU and albumen height than the control treatment (Table 2, quadratic, $P < 0.01$). However, no difference was observed on the albumen pH, relative weight of total albumen, thick albumen and thin albumen among treatments at the end of the experiment ($P > 0.05$).

Albumen Gels Characteristics

As shown in Table 3, albumen gel strength, hardness, gumminess, and chewiness decreased in a quadratic manner ($P < 0.05$) with increasing TP concentration in the diets, with minimal values obtained with 400 mg/kg TP adding group. There were no effects of increasing TP on the adhesiveness, springiness, cohesiveness, and resilience value of albumen gel ($P > 0.05$).

Egg Antioxidant Substances and Activities of Egg White

The albumen T-AOC, RP, and ORAC values increased in a quadratic manner as the dietary TP supplementation levels increased, with the highest value of RP and ORAC found in 400 mg/kg TP treatment (Figure 1; quadratic effect, $P < 0.05$). Moreover, the MDA content in albumen was also reduced in a quadratic manner, and lowest MDA content was also observed in TP400 treatment layers (quadratic effect,

Table 2. Effect of TP on the Egg White Quality of Laying Hens.[1]

Items	TP levels						*P*-value	
	Control	TP200	TP400	TP600	TP800	TP	Linear	Quadratic
pH	8.41	8.24	8.33	8.37	8.29	0.64	0.77	0.53
Albumen height, mm	7.09 ± 0.80[b]	7.21 ± 0.39[a,b]	7.63 ± 0.70[a]	7.31 ± 0.53[a]	7.33 ± 0.23[a,b]	0.02	0.06	<0.01
Haugh unit	83.82 ± 0.67[c]	84.97 ± 0.98[b]	87.13 ± 1.15[a]	85.13 ± 1.07[a–c]	85.98 ± 1.05[a,b]	<0.01	0.06	<0.01
Albumen weight, %	59.49 ± 2.37	60.02 ± 1.15	60.09 ± 0.99	61.31 ± 1.20	61.08 ± 1.33	0.21	0.02	0.08
Thick albumen weight, %	41.90 ± 7.38	43.33 ± 2.86	46.11 ± 5.48	44.62 ± 6.21	43.29 ± 4.26	0.73	0.56	0.42
Thin albumen weight, %	43.98 ± 4.45	45.22 ± 3.94	46.67 ± 5.27	42.62 ± 3.07	44.22 ± 4.38	0.57	0.70	0.72

[1]Each mean represents 6 replicates, with 5 eggs/replicate. Abbreviation: TP = tea polyphenols, TP200 = 200 mg/kg tea polyphenols, TP400 = 400 mg/kg tea polyphnols, TP600 = 600 mg/kg tea polyphenols, TP800 = 800 mg/kg tea polyphenols.
[a–c]Means in the same column with different letters differ significantly ($P < 0.05$).

Table 3. Effect of TP on the egg white gels texture of laying hens.[1]

Items	TP level						*P*-value	
	Control	TP200	TP400	TP600	TP800	TP	Linear	Quadratic
Strength (g)	758.48 ± 112.19[a]	740.04 ± 67.39[a]	567.47 ± 89.01[b]	745.7 ± 99.97[a]	784.87 ± 91.93[a]	0.01	0.59	0.01
Hardness (g)	934.39 ± 108.53[a]	903.1 ± 112.32[a]	706.22 ± 92.98[b]	905.96 ± 130.31[a]	948.39 ± 110[a]	0.02	0.53	0.02
Adhesiveness (g/cm)	–68.83 ± 17.38	–65.26 ± 16.18	–66.57 ± 22.82	–62.29 ± 14.53	–69.37 ± 8.1	0.96	0.24	0.51
Springiness (MPa)	0.98 ± 0.02	0.99 ± 0.001	0.98 ± 0.01	0.98 ± 0.02	0.99 ± 0.004	0.53	0.89	0.44
Cohesiveness (MPa)	0.55 ± 0.03	0.55 ± 0.01	0.55 ± 0.01	0.55 ± 0.02	0.54 ± 0.02	0.97	0.80	0.47
Gumminess (g)	530.65 ± 48.9[a]	488.41 ± 47.15[a]	388.7 ± 58.95[b]	499.1 ± 69.01[a]	516.01 ± 66.56[a]	0.01	0.49	0.03
Chewiness (mJ)	519.84 ± 45.14[a]	505.63 ± 64.59[a]	379.43 ± 55.43[b]	488.2 ± 74.02[a]	509.68 ± 63.93[a]	0.01	0.50	0.03
Resilience (MPa)	0.22 ± 0.03	0.21 ± 0.01	0.22 ± 0.01	0.21 ± 0.01	0.20 ± 0.02	0.38	0.12	0.18

[1]Each mean represents 6 replicates, with 5 eggs/replicate. Abbreviation: TP = tea polyphenols, TP200 = 200 mg/kg tea polyphenols, TP400 = 400 mg/kg tea polyphnols, TP600 = 600 mg/kg tea polyphenols, TP800 = 800 mg/kg tea polyphenols.
[a,b]Means in the same column with different letters differ significantly ($P < 0.05$).

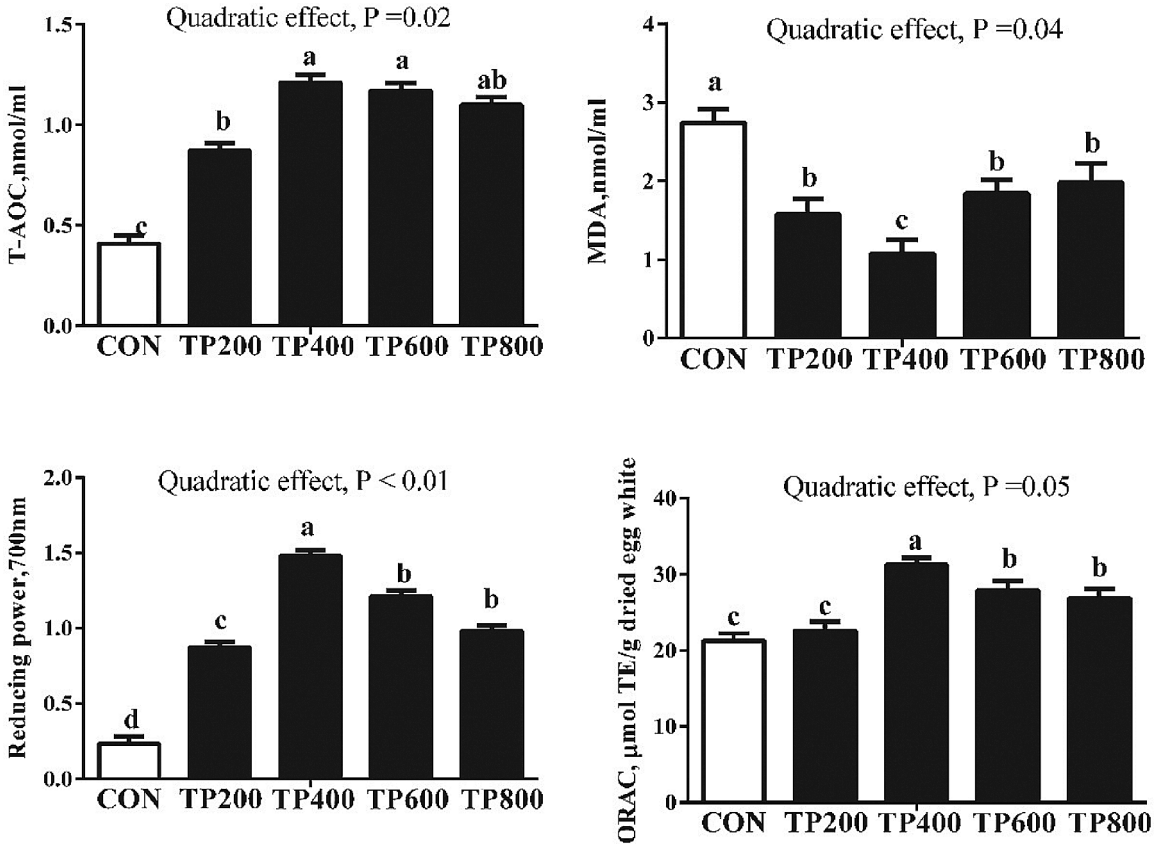

Figure 1. Effect of tea polyphenol supplementation on the antioxidant activities in egg albumen and yolk of laying hens. (A) total antioxidant capacity (T-AOC); (B) malondialdehyde (MDA) content; (C) reducing power (RP); (D) oxygen radical absorbance capacity (ORAC) value. All data are mean ± standard deviation values. Different letters significant differences between samples of different treatment. Each mean represents 6 replicates, with 4 eggs/replicate.

Table 4. Effect of TP on the egg white amino acid content of laying hens (mg/g egg white).[1]

Items	TP levels						*P*-value	
	Control	TP200	TP400	TP600	TP800	TP	Linear	Quadratic
Crude protein,%	10.1 ± 0.7	10.3 ± 0.4	10.2 ± 0.5	10.2 ± 0.7	10.2 ± 0.8	0.47	0.63	0.77
Lysine	8.0 ± 0.4	8.1 ± 0.4	7.9 ± 0.3	8.2 ± 0.5	8.1 ± 0.6	0.51	0.44	0.37
Leucine	12.4 ± 0.9	12.1 ± 0.8	11.7 ± 0.7	12.0 ± 0.7	12.1 ± 0.9	0.14	0.25	0.84
Valine	8.1 ± 0.6	8.3 ± 0.7	8.2 ± 0.8	8.4 ± 0.6	8.1 ± 0.7	0.34	0.48	0.34
Isoleucine	6.8 ± 0.4	6.5 ± 0.5	6.9 ± 0.5	6.7 ± 0.5	6.9 ± 0.3	0.44	0.19	0.37
Phenylalanine	8.5 ± 0.6	8.6 ± 0.3	8.1 ± 0.3	8.0 ± 0.4	8.0 ± 0.3	0.77	0.69	0.24
Threonine	6.7 ± 0.3	6.3 ± 0.2	6.9 ± 0.3	7.1 ± 0.3	6.5 ± 0.4	0.37	0.54	0.18
Methionine	5.4 ± 0.2	5.7 ± 0.2	5.5 ± 0.3	5.9 ± 0.3	5.4 ± 0.2	0.79	0.85	0.32
Histidine	3.0 ± 0.7	2.8 ± 0.7	3.1 ± 0.7	3.2 ± 0.7	3.1 ± 0.7	0.47	0.25	0.27
Glutamic acid	14.3 ± 0.9	12.9 ± 1.1	13.8 ± 1.2	14.8 ± 0.9	14.2 ± 1.0	0.69	0.34	0.44
Tryptophan	2.3 ± 0.1	2.5 ± 0.2	2.9 ± 0.4	2.8 ± 0.4	2.7 ± 0.2	0.24	0.88	0.77
Serine	9.7 ± 0.8	10.1 ± 0.8	9.9 ± 0.7	9.8 ± 0.6	9.8 ± 0.7	0.88	0.61	0.68
Aspartic	13.5 ± 0.8	13.4 ± 0.9	12.9 ± 0.9	12.8 ± 1.2	13.1 ± 1.2	0.27	0.49	0.19
Arginine	8.1 ± 0.6	8.4 ± 0.7	8.7 ± 0.6	8.4 ± 0.4	8.5 ± 0.4	0.63	0.33	0.69
Tyrosine	4.1 ± 0.3	4.0 ± 0.2	4.2 ± 0.2	4.4 ± 0.2	4.2 ± 0.2	0.24	0.29	0.31
Glycine	5.0 ± 0.3	5.1 ± 0.4	5.2 ± 0.3	5.5 ± 0.3	5.4 ± 0.4	0.37	0.34	0.29
Proline	4.1 ± 0.3	3.9 ± 0.2	3.8 ± 0.2	4.1 ± 0.3	4.1 ± 0.2	0.29	0.66	0.11
Cysteine	2.7 ± 0.1	2.1 ± 0.1	2.5 ± 0.1	2.6 ± 0.1	2.8 ± 0.2	0.66	0.79	0.97
ΣEAA	58.8 ± 3.5	58.4 ± 2.7	58.3 ± 1.8	59.5 ± 2.1	58.2 ± 2.6	0.54	0.81	0.47
ΣTAA	123.0 ± 3.7	120.8 ± 4.6	121.7 ± 5.2	124.7 ± 3.5	123.4 ± 3.4	0.37	0.27	0.93

[1]Each mean represents 6 replicates, with 4 eggs/replicate. Abbreviation: TP = tea polyphenols, TP200 = 200 mg/kg tea polyphenols, TP400 = 400 mg/kg tea polyphenols, TP600 = 600 mg/kg tea polyphenols, TP800 = 800 mg/kg tea polyphenols, EAA = percentage of essential amino acid for humans (including lysine, leucine, valine, isoleucine, phenylalanine, threonine, methionine, and histidine), TAA = percentage of total amino acids.

$P < 0.05$). As shown in Table 4, there were no differences in each amino acid concentration in albumen among all experimental treatments ($P > 0.05$).

Overview of the Quantitative Proteomics

With high throughput mass spectrometry, 258 proteins were definitively identified in egg white from the 776 unique peptides in the present study. According to the protein molecular weight (MW) distribution, the majority of the MWs ranged from 10 to 200 kDa. The MWs of 15 proteins were >200 kDa, and 6 proteins were <10 kDa (Figure 2A). Additionally, most of the identified peptides had good peptide coverage, with 46% proteins having >10% of the sequence coverage, and 23% proteins having >20% sequence coverage (Figure 2B).

A total of 31 differentially expressed proteins that changed by more than 1.2-fold were observed between the egg albumen from hens fed the TP400 diet and those fed the basal diet (Table 5). Compared to the control group, 19 proteins were upregulated, while 12 proteins were downregulated in the TP400 group. To better understand the differentially expressed proteins between the control and TP400 groups, the Uniprot database and GO were used for the annotation of 31 proteins. After analysis of molecular function, we found that the protein participated in response to stimulus (16%), metabolic process (13%) and biological regulation (12%) were differential expressed (Figure 3A). Additionally, 37, 20, and 17% differential proteins located in the extracellular, cell and membrane, respectively (Figure 3B). In the molecular function category, the differentially expressed protein that are binding proteins (66%) followed by catalytic activity (13%), suggesting that the relevant function were important of TP function in egg albumen.

GO Annotation and KEGG Analysis

Moreover, the GO annotation and KEGG pathway enrichment analysis were used to determine overrepresented biological events and to provide a primary overview of the albumen proteome influence by TP400. It showed that 9 immune response proteins (P10184, R4GI90, P01875, Q6IV20, Q64EU6, P02701, P08110, P0CB50, A0A1D5PQ63), 11 binding proteins (A0A1D5PZE3, F1NTQ2, Q7SX63, F1NRV5, P24802, A0A1L1RM02, E1BTX1, A0A1L1RMF4, A0A1D5P1N3, A0A1L1RML6, A0A1L1RQF3), and 3 antioxidant activity protein (P0CB50, P20136, Q8JG64) were changed in albumen of laying hens fed TP400 (Table 5). The upregulated proteins were shown enriched in identical protein binding and transferase activity, while the downregulated protein were in carbohydrate binding (Figure 4). The differentially expressed proteins mainly involved in pyruvate metabolism and cysteine and methionine metabolism, glutathione metabolism, glycolysis, and protein processing in endoplasmic reticulum pathway (Table 6).

DISCUSSION

Oxidative stress is an imbalance between the free radicals and antioxidants at either the cellular or the individual level (Sies, 2015). It is said that for high-productive commercial laying hens, they suffered severe

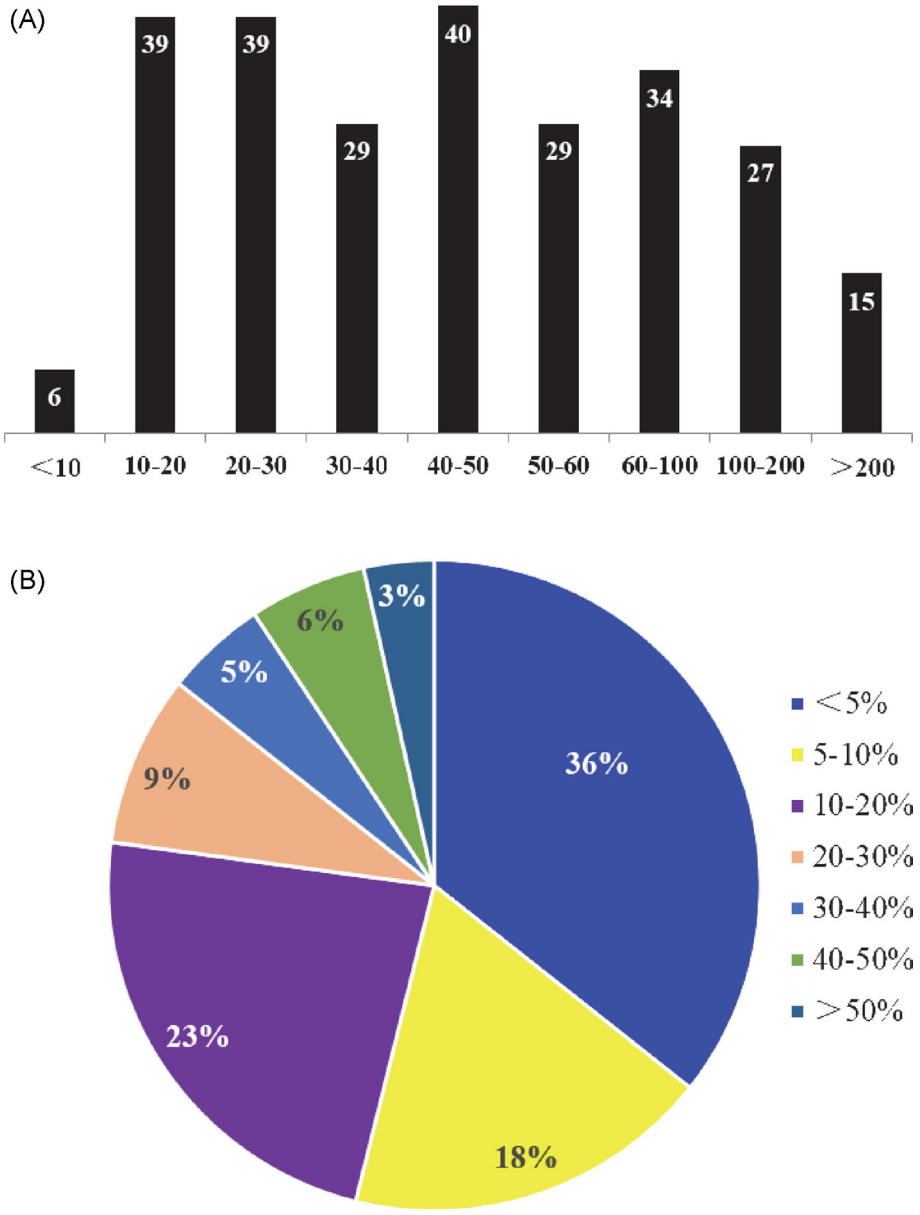

Figure 2. Molecular weight (MW) distribution and coverage in this study. (A) Distribution of protein identified among different MWs. (B) Coverage of protein by the identified peptides. Most of the identified peptides had good peptide coverage, with ∼46% proteins having >10% of the sequence coverage, and ∼23% proteins having >20% sequence coverage. Each mean represents 6 replicates, with 2 eggs/replicate.

oxidative stress, especially during the egg-peaking and late-laying stage (Afzal et al., 2015). Green TP have antioxidant properties in light of their ability to scavenge reactive oxygen, reactive nitrogen species and chelating redox active transition metal ions resulting in an anti-inflammatory effect (Uesato et al., 2001; Frei and Higdon, 2003; Zhen et al., 2007). In present study, we found that TP supplementation at 400 mg/kg was effective in improving egg albumen (egg white) quality after 8 wk of feeding. Similarly, in previous studies, the green tea or its polyphenol extracts were found to improve egg albumen quality in layers during an oxidative stress challenge (Biswas et al., 2000; Biswas and Wakita, 2001; Afzal et al., 2015; Yuan et al., 2016; Wang et al., 2018). However, the mechanism involved in TP-mediated improvement effect and the proteomic alternations in egg whites are still unclear.

We observed 31 differentially expressed proteins between the dietary absence and presence of TP400 using a TMT-based quantitative proteomic analysis, with 19 of them were upregulated and 12 were downregulated. Among them, the expression level of ovalbumin (OVA)-related Y protein (I0J179) and ovoinhibitor (P10184) was higher after TP addition. This result is in agreement with our previous study, in which we found that EGCG can increase the level of OVA-related Y protein and decreased the OVA-related X protein under oxidative stress model induced by vanadium (Wang et al., 2017). The free sulfhydryl and high hydrophobicity properties of OVA

Table 5. Differentially expressed proteins in albumen of 400 mg/kg TP group versus control group.[1]

Protein accession[2]	Description	Description of biological function	Gene	FC	*P*-value
Egg white protein					
I0J179	Ovalbumin-related Y	None predicted	JPH3	1.60	<0.01
P10184	Ovoinhibitor	Protease binding	OIH	1.39	0.01
Cell plasma membrane related protein					
F1NTQ2	Beta-hexosaminidase subunit beta	Lysosome organization	HEXB	1.43	0.05
A0A1D5NYL9	Uncharacterized protein	None predicted	LUZP2	1.33	0.01
F1NRV5	Uncharacterized protein	Immune response	CD80	0.76	0.02
Protein related to biological process inside the cell metabolic process					
Response to stimulus					
Q7SX63	Heat shock 70 kDa protein	Response to stimulus, binding	HSPA8	3.55	0.01
P08110	Endoplasmin	Response to stimulus, binding	HSP90B1	2.41	0.02
P0CB50	Peroxiredoxin-1	Cell redox homeostasis	PRDX1	1.75	0.04
R4GI90	Uncharacterized protein	Innate immune response	OvoDA3	1.53	0.01
Q6IV20	Gallinacin-11	Defense response to bacterium	GAL11	1.26	0.04
P20136	Glutathione S-transferase 2	Cell redox homeostasis	GSTM2	1.25	0.04
R4GLT1	Cystatin	Defense response	CST3	1.24	0.04
P02701	Avidin	Antibacterial response; biotin binding	AVD	1.24	0.01
Q8JG64	Protein disulfide-isomerase A3	Cell redox homeostasis, identical protein binding	PDIA3	1.22	0.01
A0A1D5PQ63	Uncharacterized protein	Immune response	ENPP2	0.75	0.03
A0A1L1RMF4	Uncharacterized protein	Immune response	IGLL1	0.59	0.02
P01875	Ig mu chain C region	Immune response	—	0.49	0.04
A0A1L1RML6	Uncharacterized protein	Immune response	IGLL1	0.45	0.04
A0A1L1RQF3	Immunoglobulin heavy variable 3-48	Immune response	IGHV3-48	0.41	0.02
Cell organization or biogenesis					
Q64EU6	Betacellulin	Cell proliferation	BTC	1.39	0.01
E1BTX1	Lipocalin	None predict	PTGDS	0.73	0.01
A0A1D5P5J0	Chromatin target of PRMT1 protein	Cell proliferation	CHTOP	0.63	0.02
Metabolic process					
Q5ZME2	Malate dehydrogenase, cytoplasmic	Carbohydrate metabolic process	MDH1	2.30	0.01
P00940	Triosephosphate isomerase	Gluconeogenesis	TPI1	2.16	0.02
P00340	L-lactate dehydrogenase A chain	Carbohydrate metabolic process	LDHA	1.71	0.03
O57579	Aminopeptidase Ey	Peptide and metal binding	ANPEP	1.26	0.03
A0A1D5PZE3	Apolipoprotein A-I	Lipid binding	APOA1	1.22	<0.01
A0A1D5P1N3	Uncharacterized protein	ATP binding	PRKG2	0.78	0.01
P24802	Procollagen-lysine,2-oxoglutarate 5-dioxygenase 1	L-ascorbic acid binding	PLOD1	0.71	0.02
R4GJP9	Histone H2A type 1-J	DNA binding	HIST1H2AJ	0.24	0.03
A0A1L1RM02	Uncharacterized protein	GTP binding	EEF1A	0.07	0.03

[1]Each mean represents 3 replicates, with 2 eggs/replicate.
[2]Protein accession number from Uniprot database (www.Uniprot.org), FC = fold change.

might affect the liquidation of thick egg white protein (Smith et al., 2000), therefore increasing the albumen height and HU of eggs. Polyphenols are reported to bind with proteins and metals, such as lysozyme, ovomucin, OVA, gelatin, and bovine serum albumin (Oda et al., 1998; Bohin et al., 2013). Also, we found that TP changed the binding protein expression levels (A0A1D5PZE3 [apolipoprotein A-I], F1NTQ2 [beta-hexosaminidase subunit beta], Q7SX63 [heat shock 70 kDa protein], F1NRV5 [uncharacterized], P24802 [procollagen-lysine], A0A1L1RM02 [uncharacterized], E1BTX1 [lipocalin], A0A1L1RMF4 [uncharacterized], A0A1D5P1N3 [uncharacterized], A0A1L1RML6 [uncharacterized], A0A1L1RQF3 [immunoglobulin heavy variable 3-48]). Moreover, previous studies have suggested that the structure of these proteins can be changed by the binding of polyphenols (Yuksel et al., 2010; Kanakis et al., 2011). Ognjenović et al. (2014) demonstrated that the addition of EGCG induced an increase in OVA β-sheet structure content to affect the secondary structure of OVA through a conformational change. Wang et al. (2018) found that supplementation of TP in layer's diet increased ovomucin fraction. On the other hand, the gel properties of albumen can reflect the texture of albumen. In our research, we found that TP supplementation could significantly decrease strength, hardness, gumminess, and chewiness of albumen gels. Albumen gelling properties are attributed to several factors, including pH, heating temperature and time, protein level and ionic strength (Hammershøj et al., 2010). Reduction of gel strength, hardness, gumminess, and chewiness from the addition of TP may be attributed to the main component of TP, catechin, which has chelating effect on metal ions, such as Fe^{2+}, Cu^{2+}, Zn^{2+}, and Ca^{2+} (Brown et al., 1998; Coudray et al., 1998; Ohyoshi et al., 1999), which influence hydrogen bonds and electrostatic interactions of albumen gel (Zhang et al., 2015).

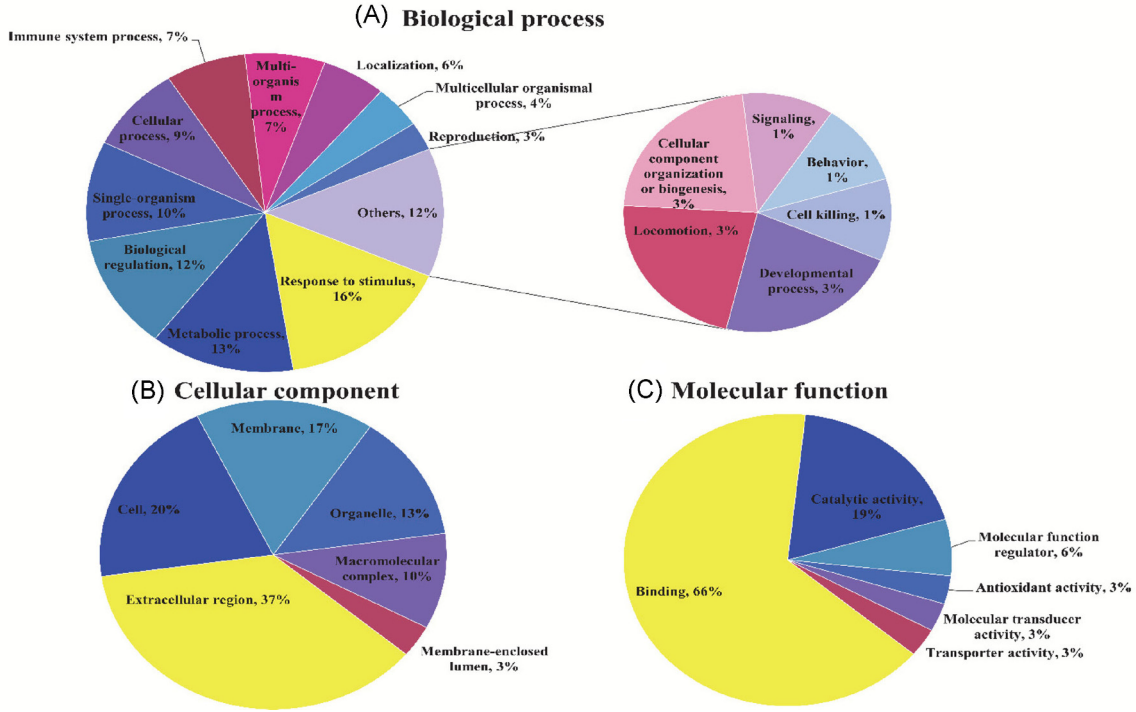

Figure 3. Bioinformatics analysis of the 31 proteins (1.2-fold) that were differentially expressed between the control and 400 mg/kg tea polyphenol groups. (A) Biological process: the protein participated in response to stimulus (16%), metabolic process (13%), and biological regulation (12%) were differential expressed; (B) cellular component: 37, 20, and 17% differential proteins located in the extracellular, cell and membrane, respectively; and (C) molecular function: binding proteins (66%) and catalytic activity (19%) were ranked at the top of the category for differential protein. Each mean represents 6 replicates, with 2 eggs/replicate.

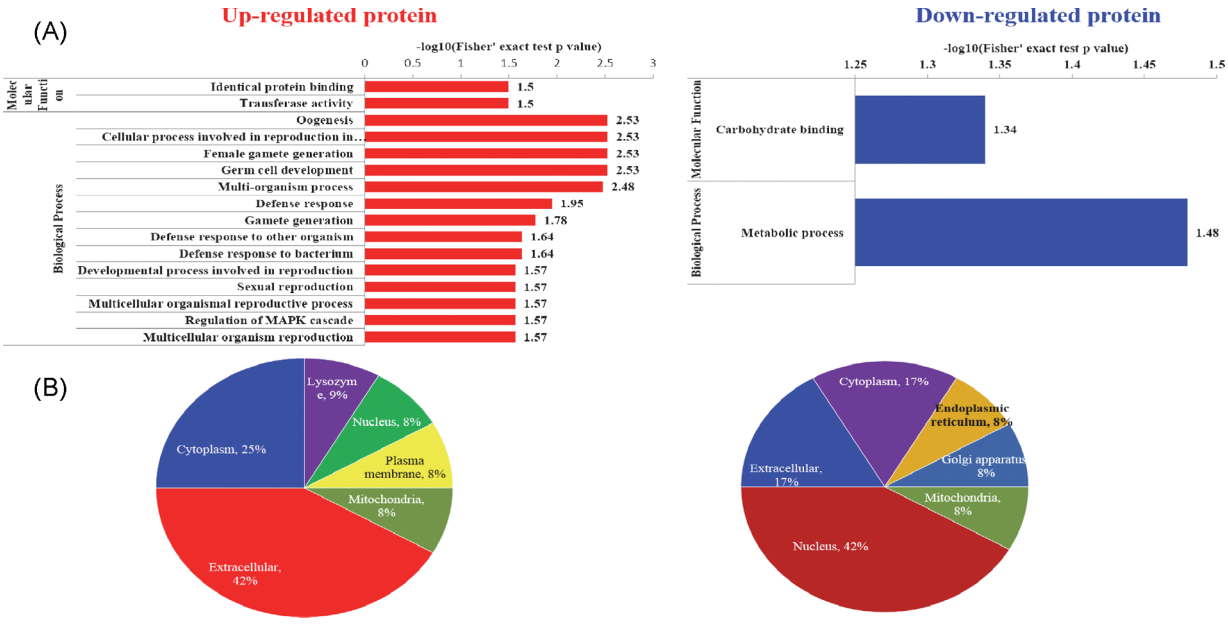

Figure 4. Functional classification of the significant up-regulated and down-regulated protein. (A) Molecular function and biological process; (B) subcellular location. Each mean represents 6 replicates, with 2 eggs/replicate.

Eggs are considered as a good source of dietary antioxidants (Nimalaratne et al., 2011; Nimalaratne and Wu, 2015). The DPPH radical-scavenging capacity assay, RP and the ORAC assay are the mainly methods used to evaluate antioxidant effects (McDonald-Wicks et al., 2006). In this study, we observed that both the RP and ORAC were improved by dietary TP400 supplementation. We also noted that dietary TP resulted in lower MDA and higher T-AOC in egg white. These observations suggest that the antioxidant capacity of

Table 6. Enriched KEGG pathway-based sets and GO terms of proteins of differential abundance in the albumen from layers fed 400 mg/kg tea polyphenols.[1]

KEGG term	Term	Count	Protein accession	*P* value[2]
gga00620	Pyruvate metabolism	2	P00340, Q5ZME2	0.01
gga00270	Cysteine and methionine metabolism	2	P00340, Q5ZME2	0.01
gga00480	Glutathione metabolism	2	P20136, O57579	0.02
gga00010	Glycolysis/gluconeogenesis	2	P00940, P00340	0.04
gga04141	Protein processing in endoplasmic reticulum	3	Q7SX63, P08110, Q8JG64	0.04

[1]Each mean represents 3 replicates, with 2 eggs/replicate.
[2]*P* values are calculated according to a modified Fisher's exact test and corrected for multiple testing using the Bonferroni correction provided by DAVID.

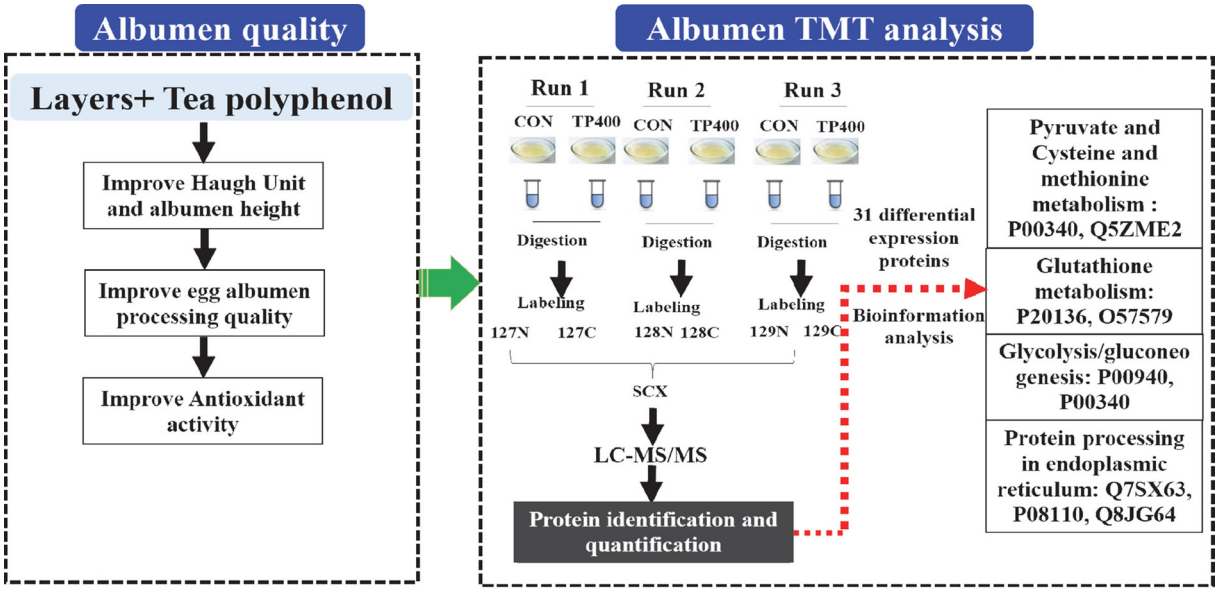

Figure 5. The overview of effect of EGCG in layers. Tea polyphenol (TP) improve albumen quality and antioxidant activity, and the improve mechanism of TP on albumen quality may act through regulating binding mediation, immune function and antioxidant activity-related proteins.

eggs was improved by TP supplementation. In agreement with our observations are the findings reported by Wang et al. (2018) who found that TP decreased the protein carbonyl content of albumen. Moreover, it was observed that TP enhanced the ovomucin content in egg white. Recently, it has been demonstrated that egg proteins, peptides, aromatic amino acids (tryptophan and tyrosine), phospholipids, vitamin E (α-tocopherol), carotenoids, and phosvitin are the main compounds responsible to the egg antioxidant activities (Dávalos et al., 2004; Hargitai et al., 2006; Katayama et al., 2006; Liu et al., 2015). In current study, we did not find that dietary TP increased the tryptophan in egg white, which may have contributed to the higher antioxidative capacity of eggs. It could be argued that the abundance of hydrophobic amino acids might not be the only reason that led to the enhanced antioxidant capacity of the eggs. Further studies, however, are required to clarify the precise mechanism of action for this effect. As a strong antioxidant, TP has the capability to scavenge free radicals due to its hydroxyl groups on the B and D rings of the polyphenol molecule. Additionally, its metal-chelating properties also may be attributed to the antioxidative activity. Indeed, 3 proteins involved in antioxidative function (P0CB50 [peroxiredoxin-1], P20136 [glutathione S-transferase 2], Q8JG64 [protein disulfide-isomerase A3]) were upregulated in current study. Also, we found that the differentially expressed proteins induced by TP mainly enriched in the carbohydrate metabolism signaling pathway (pyruvate metabolism and glycolysis) and redox homeostasis pathway in KEGG analysis (cysteine and methionine metabolism, glutathione metabolism, and endoplasmic reticulum pathway). Green tea consumption resulted in enhanced enzyme activities of carbohydrate metabolism and antioxidant defenses system, which may lead to improved health of humans and animal (Khan et al., 2007; Sundaram et al., 2013). This may indicate that the TP can increase the activities of certain enzymes of glucose degradation and its synthesis. Also, this effect may partially be related to its antioxidant free radical scavenging properties that lower oxidative damage. Our results also indicated that the expression of P20136 and P0CB50, which is related to the regulation of cell redox homeostasis, was higher induced by the TP addition. On the other hand,

polyphenols have been demonstrated to modulate the inflammatory process and stimulators via several individual and synergistic mechanisms (Tipoe et al., 2007; Afzal et al., 2015; Magrone and Jirillo, 2018). We also observed that 11 immune response proteins (P10184 [ovoinhibitor], R4GI90 [uncharacterized], P01875 [Ig mu chain C region], Q6IV20 [gallinacin-11], Q64EU6 [betacellulin], P02701 [avidin], P08110 [endoplasmin], P0CB50 [peroxiredoxin-1], A0A1D5PQ63 uncharacterized]) were altered by TP. Thus, the TP addition can regulate the expression of proteins related to cell redox and immune response.

CONCLUSION

As a result of the present experiment, supplementation of a laying hen diet with TP has beneficial effects on egg weight, HU, albumen height, strength, hardness, gumminess and chewiness of albumen gel. The improving mechanism of TP on albumen quality may act through regulating binding mediation and antioxidant activity-related proteins (Figure 5).

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