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Research Article

Correlation analysis between fertilized hen egg yolk extract and lipid metabolism

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Abstract

Looking for safe and effective dietary substitutes with high nutritional value has always been a hot spot in changing human dietary structure, we studied the protein components in fertilized hen egg yolk extract (FEYE) from lipid metabolism. LC-MS/MS analysis was performed on FEYE, and Western Blot (WB) was used to verify the protein expression of the lipid metabolism-related proteins ApolipoA-1 (APOA1) and Bile salt-activated lipase (BSAL, CEL). A total of 294 proteins were identified based on LC-MS/MS; after analysis and screening, the results showed that the metabolic pathway and PPAR signalling pathway were the main biological processes affecting lipid metabolism in FEYE. WB results showed that APOA1 and CEL were expressed in FEYE, which was consistent with LC-MS/MS results. We believe that FEYE is associated with lipid metabolism. At the same time, this study provides a basis for further study of proteomics of FEYE and a new idea for the study of food ingredients.

Keywords: FEYE, LC-MS/MS, Lipid metabolism

Highlights

- Comprehensive proteomic sequencing of fertilized egg yolks
- Starting from lipid metabolism and verification of fertilized egg yolk
- New ideas for dietary substitutes based on fertilized egg yolks

INTRODUCTION

Egg is an important part of human diet; its perfect properties are derived from its ideal amino acid composition, high nutritional value and considerable biodiversity (Lesnierowski and Stangierski, 2018). Most eggs on the market are unfertilized, while in some Asian countries, fertilized eggs are traditionally considered a natural dietary supplement. The protein metabolism of fertilized hen egg is very strong, egg yolk as the main site of fertilization, the contents of protein, total amino acid and free amino acid gradually increase post fertilization. The nutritional level of fertilized hen egg is much higher than that of ordinary eggs (Zhang *et al.*, 2010) and has the biological activity to support the development of new life (Wang and Wu, 2014). Fertilized hen eggs help to increase appetite and enhance immune function, especially for pregnant women and the elderly, according to Compendium of Materia Medica, China's most famous pharmacopoeia.

With the change in lifestyle in modern society, high-fat and high-sugar foods are frequently consumed, and the proportion of obese people is gradually increasing; this leads to increased levels of lipid metabolism-related diseases such as diabetes, hyperlipidemia and hypertension (Cheong and Xu, 2021). Using diet to change fat accumulation has become a hot spot. A recent study suggests that eggs can be part of a healthy diet for the general population and people at risk of cardiovascular disease (Fuller *et al.*, 2015). The World Health Organization reported that if fat and meat intake were controlled, there was no need to limit egg yolk intake (WHO, 2003). Studies in South Korea have also shown that eating eggs has a

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positive effect on metabolic syndrome (Park *et al.*, 2018). Egg is a natural perfect food; egg yolk contains a lot of carotenoids, such as lutein and zeaxanthin (Karadas *et al.*, 2006), resulting in yellow pigment in egg yolks, these antioxidant compounds have also been proven to be beneficial to health (Mares-Perlman *et al.*, 2002) and the nutritional value of fertilized hen eggs is much higher than that of ordinary eggs. Therefore, this knowledge can be used to develop products based on fertilized hen eggs for specific application. Peroxisome proliferator activated receptor (PPAR) is a ligand-activated transcription factor which has a wide range of physiological functions in animals. It can regulate physiological processes such as lipid metabolism through the expression of related genes (Wagner and Wagner, 2020). Metabolic pathways are a series of chemical reactions catalyzed by enzymes in cells, and lipid metabolism is also regulated by metabolic pathways. Therefore, we screened genes related to lipid metabolism by studying the related genes in PPAR signaling pathway and metabolic pathway. This study systematically introduced the protein components in fertilized hen egg yolk, which not only provided a new idea for improving human dietary components to prevent hyperlipidemia, hypertension and hyperglycemia but also provided a basis for future research on lipid metabolism of fertilized hen egg yolk.

MATERIALS AND METHODS

Separation of egg yolk extract from fertilized hen eggs (FEYE): Separate the whole egg yolk of the fertilized hen egg, pick the egg yolk film with a needle, and carefully collect the egg yolk in the centrifuge tube with a syringe. Add PBS (Yolk: PBS = 7:3) to the centrifuge tube, centrifuge the egg yolk suspension at 3340 g for 20 min at 4°C, collect the precipitate, and store at 4°C.

FEYE preparation and In-gel digestion: The above samples were diluted and added with 10 mM DTT for 1 h at 56°C, 55 mM iodoacetamide was added at room temperature for 45 min. Cleaned with 100 µL of 25 mM NH_4HCO_3 for 10 min, dehydrated with 25 mM NH_4HCO_3 and 50% acetonitrile twice, and dried in a vacuum. Then 12.5 ng/µL of trypsin was added overnight at 37°C. Trypsin peptide was extracted from the gel block, and the appropriate volume was 60% acetonitrile and 0.2% TFA. Scroll 20 min, ultrasonic 5 min, and take the supernatant to save. After evaporating acetonitrile in SpeedVac, the sample was desalted by C18 ZipTip (Millipore), and half of the eluent was analyzed with LC-MS/MS.

HPLC-MS/MS analysis: The samples were resuspended with 30 µL solvent C (C: water with 0.1% formic acid), separated by nanoLC and analyzed by on-line electrospray tandem mass spectrometry. The experiments were performed on a Nano ACQUITY UPLC system (Waters Corporation, Milford, MA) connected to a Q-Exactive mass spectrometer (Thermo Fisher Scientific, MA, USA) equipped with an online nano-electrospray ion source. 10 µL peptide sample was loaded onto the trap column (Thermo Scientific Acclaim PepMap C18, 100 µm × 2 cm), with a flow of 10 µL/min for 3 min and subsequently separated on the analytical column (Acclaim PepMap C18, 75 µm × 15 cm) with a 90 min linear gradient, from 5% D (D: ACN with 0.1% formic acid) to 55% D. The column was re-equilibrated at initial conditions for 10 min. The column flow rate was maintained at 300 nL/min. The electrospray voltage of 2 KV versus the inlet of the mass spectrometer was used.

The mass spectrometer was run under data dependent acquisition mode, and automatically switched under MS and MS/MS mode. MS1 mass resolution was set as 70 K with m/z 300-1800, and MS/MS resolution was set as 17.5 K under HCD mode. The dynamic exclusion time was set as 10 seconds.

Database searching parameters: Tandem mass spectra were processed by PEAKS Studio version 8.5 (Bioinformatics Solutions Inc,

Waterloo, Canada). PEAKS DB was set up to search the UniProt-gallus gallus database (ver 201708, 29725 entries) assuming trypsin as the digestion enzyme. PEAKS DB was searched with a fragment ion mass tolerance of 0.05 Da and a parent ion tolerance of 7 ppm. Carbamidomethylation (C) was specified as the fixed modification. Oxidation (M), Deamidation (NQ), and Acetylation (Protein N-term), were specified as the variable modifications. Peptides were filtered by 1% FDR and 1 unique peptide.

Blast2GO version 4 was used for functional annotation. Whole protein sequence database was analyzed by BlastP using whole database and mapped, annotated with gene ontology database. Statistically altered functions of different expressed proteins was calculated by Fisher's exact test in BLAST2GO ($P<0.05$). Pathway analysis was processed by KOBAS (http://kobas.cbi.pku.edu.cn/).

SDS-PAGE: Gradient dilution of the above samples with PBS. The total protein loading quantity of each sample was 30 μg and heated at 95°C for 10 min. Each gradient diluted sample was subjected to SDS-PAGE electrophoresis and run at 80 V until the samples reached the front of the gel. Gels were stained using Coomassie Brilliant blue G-250 (Solarbio, Beijing, China). Images were acquired by the Gel Doc XR imaging system and analyzed by the software Image Lab (All Bio-Rad, California, America).

Western Blot: The samples were subjected to SDS-PAGE electrophoresis according to the above method and then transferred to the nitrocellulose membrane (Boster, Wuhan, China). The membranes were blocked with 5% non-fat dried milk in Tris-Buffered Saline-Tween (TBST) for 1 h. The rabbit polyclonal antibodies include CEL (Bioss, Beijing, China, bs-13830R, 1:1000), APOA1 (Bioss, Beijing, China, bs-0849R, 1:1000) primary antibodies were added for incubation overnight at 4°C. The sample was shaken and washed three times with TBST (10 min each) and then incubated with horseradish peroxidase-conjugated secondary antibodies (ComWin, Beijing, China, CW0156, 1:10,000) for 1h at 37°C thermostatic Shaker. Then shaken and washed six times with TBST (5 min each). Finally, immunoreactive signals were developed using ECL Western blot detection reagents (ComWin, Beijing, China, CW0049S).

Statistical methods: The data were presented as means ± SD. SPSS 17.0 software (IBM, Armonk, NY, USA) was used for statistical analysis. While, comparisons between 2 groups were performed by unpaired Student's test. Statistical significance was defined as $P<0.05$.

RESULTS

Protein profiling: In order to analyze the types of protein components in FEYE, 294 proteins were identified by LC-MS/MS, based on standard (FDR < 1% and unique peptide numbers ≥ 2). Detailed information on all identified proteins is available in Table 1. According to statistics, only two proteins (Ovalbumin, Vitellogenin-2) accounted for 90-100% of the total amino acids in all proteins, and there were 5, 8, 18, 15 proteins in 80-89%, 70-79%, 60-69%, 50-59%, respectively (Fig. 1A). There were 137 proteins (Fig. 1B) with the number of specific peptides greater than or equal to 2 in the protein, and two proteins greater than or equal to 100 were Vitellogenin-2 and Apolipoprotein B, respectively. To sum up, this protein sequencing results are reliable.

GO analysis: GO analysis classified the identified proteins based on Go terms; proteins detected are marked as biological processes, molecular functions and cellular components. The top 20 paths with the largest number of proteins were selected for the study; the classification results were summarized in Fig. 2, the most enriched biological processes are signal transduction (GO: 0007165), proteolysis (GO: 0006508) and lipid transport (GO: 0006869), which contain 18, 10 and 9 proteins, respectively. From the perspective of lipid transport, apolipoprotein and luteinizing

Table 1. List of all identified FEYE proteins

Protein ID	Uni Prot Entry	Coverage (%)	Unique	Protein description
2	P01012	92	98	Ovalbumin OS=Gallus gallus PE=1 SV=2
3	A0A1D5PV37	90	7	Vitellogenin-2 OS=Gallus gallus PE=4 SV=1
4	A0A1D5NW68	87	9	Serum albumin OS=Gallus gallus PE=3 SV=1
5	A0A1D5P4L7	81	5	Ovotransferrin OS=Gallus gallus PE=3 SV=1
6	P02789	79	4	Ovotransferrin OS=Gallus gallus PE=1 SV=2
7	F1NFL6	80	186	Vitellogenin-2 OS=Gallus gallus PE=4 SV=1
11	F1NV02	72	527	Apolipoprotein B OS=Gallus gallus PE=4 SV=2
12	Q98UI9	68	4	Mucin-5B OS=Gallus gallus PE=1 SV=1
13	A0A1D5NUW2	75	13	Vitellogenin-1 OS=Gallus gallus PE=4 SV=1
15	P87498	73	5	Vitellogenin-1 OS=Gallus gallus PE=1 SV=1
16	F1NZY2	68	4	Uncharacterized protein OS=Gallus gallus PE=4 SV=1
18	P11682	66	4	Apolipoprotein B (Fragment) OS=Gallus gallus PE=2 SV=1
19	A0A1D5P9F9	61	85	Uncharacterized protein OS=Gallus gallus PE=4 SV=1
23	A0A1D5P2X2	63	95	Uncharacterized protein OS=Gallus gallus PE=4 SV=1
25	F1NK40	38	48	Uncharacterized protein OS=Gallus gallus PE=4 SV=4
26	A0A1D5P6F4	23	36	Uncharacterized protein OS=Gallus gallus PE=4 SV=1
28	A0A1D5PCH3	20	41	Uncharacterized protein OS=Gallus gallus PE=4 SV=2
29	A0A1L1S0P1	65	20	Uncharacterized protein OS=Gallus gallus PE=4 SV=1
30	F1NWX6	57	33	Plasminogen OS=Gallus gallus PE=3 SV=2
32	A0A1D5PAL0	38	41	Uncharacterized protein OS=Gallus gallus PE=4 SV=1
35	A0A1D5NVH7	21	2	Uncharacterized protein OS=Gallus gallus PE=4 SV=1
36	A0A1D5PZS5	24	1	Uncharacterized protein OS=Gallus gallus PE=4 SV=1
37	A0A1L1RV31	43	34	Prothrombin OS=Gallus gallus PE=3 SV=1
39	Q02020	57	23	Fibrinogen beta chain (Fragment) OS=Gallus gallus PE=1 SV=1
41	A0A1L1RJ91	34	3	Uncharacterized protein OS=Gallus gallus PE=4 SV=1
42	H9L385	76	23	Hemopexin OS=Gallus gallus PE=4 SV=3
44	P10184	61	1	Ovoinhibitor OS=Gallus gallus PE=1 SV=2

Cont. Table 1.

Table 1., Cont. ...

Protein ID	Uni Prot Entry	Coverage (%)	Unique	Protein description
46	F1NMN2	61	1	Uncharacterized protein OS=Gallus gallus PE=4 SV=3
47	A0A1L1RUH2	26	1	Uncharacterized protein OS=Gallus gallus PE=4 SV=1
50	A0A1L1RMA4	60	10	Uncharacterized protein OS=Gallus gallus PE=4 SV=1
51	E1BV78	63	23	Uncharacterized protein OS=Gallus gallus PE=4 SV=2
52	P79762	63	25	Zonapellucida sperm-binding protein 3 OS = Gallus gallus PE=1 SV=4
54	A0A1D5PU94	26	38	Uncharacterized protein OS=Gallus gallus PE=4 SV=1
57	E1C7P4	27	25	Uncharacterized protein OS=Gallus gallus PE=4 SV=2
58	A0A1D5P531	58	11	Uncharacterized protein OS=Gallus gallus PE=3 SV=1
61	P01014	55	16	Ovalbumin-related protein Y OS=Gallus gallus PE=1 SV=1
62	A0A1D5PN61	50	22	Clusterin OS=Gallus gallus PE=3 SV=1
66	E1C7A7	43	17	Uncharacterized protein OS=Gallus gallus PE=4 SV=2
69	O42273	51	1	Protein TENP OS=Gallus gallus PE=2 SV=1
71	A0A1D5PZE3	76	30	Apolipoprotein A-I OS=Gallus gallus PE=3 SV=1
76	A0A1L1RTQ4	52	18	Uncharacterized protein OS=Gallus gallus PE=4 SV=1
82	P14448	23	15	Fibrinogen alpha chain OS=Gallus gallus PE=1 SV=4
84	A0A1L1S0R0	72	2	Uncharacterized protein OS=Gallus gallus PE=4 SV=1
85	P01013	64	4	Ovalbumin-related protein X (Fragment) OS=Gallus gallus PE=3 SV=1
87	A0A1L1RWP3	59	4	Uncharacterized protein OS=Gallus gallus PE=4 SV=1
90	Q91025	45	35	Vitellogenin-3 (Fragments) OS=Gallus gallus PE=2 SV=2
91	P01005	79	24	Ovomucoid OS=Gallus gallus PE=1 SV=1
92	E1C897	38	13	CathepsinE-A-like protein OS=Gallus gallus PE=2 SV=3
94	A0A1D5NTE9	53	1	Uncharacterized protein OS=Gallus gallus PE=3 SV=1

Cont. Table 1.

Table 1., Cont. ...

Protein ID	Uni Prot Entry	Coverage (%)	Unique	Protein description
95	P41366	66	13	Vitelline membrane outer layer protein 1 OS=Gallus gallus PE=1 SV=1
96	F1NVF3	31	15	Uncharacterized protein OS=Gallus gallus PE=3 SV=1
97	O93510	19	1	Gelsolin OS=Gallus gallus PE=2 SV=1
99	A0A1D5PH32	18	1	Gelsolin OS=Gallus gallus PE=4 SV=1
102	A0A1D5P4I3	39	1	Uncharacterized protein OS=Gallus gallus PE=3 SV=1
104	F1NA58	23	10	Uncharacterized protein OS=Gallus gallus PE=3 SV=3
105	R9PXM5	65	26	Uncharacterized protein OS=Gallus gallus PE=4 SV=2
106	F1NEQ4	9	10	Uncharacterized protein OS=Gallus gallus PE=4 SV=4
107	P00698	80	47	Lysozyme C OS=Gallus gallus PE=1 SV=1
108	P27731	81	14	Transthyretin OS=Gallus gallus PE=1 SV=1
110	A0A1D5NW85	52	8	Extracellular fatty acid-binding protein OS=Gallus gallus PE=3 SV=1
112	A0A1D5PBP6	9	9	Uncharacterized protein OS=Gallus gallus PE=3 SV=1
113	A0A1D5PD98	6	10	Uncharacterized protein OS=Gallus gallus PE=4 SV=1
117	R4GFR4	48	11	Metalloproteinase inhibitor 3 OS=Gallus gallus PE=4 SV=2
119	P02752	38	13	Riboflavin-binding protein OS=Gallus gallus PE=1 SV=2
121	A0A1L1RKY8	52	9	Uncharacterized protein OS=Gallus gallus PE=4 SV=1
124	Q5ZMQ2	35	4	Actin cytoplasmic 2 OS=Gallus gallus PE=1 SV=1
125	Q9PRS8	56	8	Ovocleidin-17 OS=Gallus gallus PE=1 SV=2
130	A0A1D5PU28	11	5	Uncharacterized protein OS=Gallus gallus PE=4 SV=1
131	A0A1D5PS90	53	7	Uncharacterized protein OS=Gallus gallus PE=4 SV=1
133	F1NIU3	10	8	Uncharacterized protein OS=Gallus gallus PE=4 SV=3
134	P01875	21	7	Ig mu chain C region OS=Gallus gallus PE=2 SV=2
135	A0A1D5P5V5	7	8	Uncharacterized protein OS=Gallus gallus PE=4 SV=1

Cont. Table 1.

Table 1., Cont. ...

Protein ID	Uni Prot Entry	Coverage (%)	Unique	Protein description
138	A0A1D5PI04	7	7	Uncharacterized protein OS=Gallus gallus PE=4 SV=1
143	A0A1D5P4L6	8	7	Uncharacterized protein OS=Gallus gallus PE=4 SV=1
146	F1NDH2	22	7	Angiotensinogen OS=Gallus gallus PE=3 SV=2
147	P02659	65	34	Apovitellenin-1 OS=Gallus gallus PE=1 SV=1
149	A0A1L1RWR0	46	7	Transthyretin OS=Gallus gallus PE=3 SV=1
150	A0A1D5PJX9	22	4	Uncharacterized protein OS=Gallus gallus PE=4 SV=1
151	A0A1D5PEU7	19	3	Uncharacterized protein OS=Gallus gallus PE=4 SV=1
153	Q8JIG5	37	12	Alpha-1-acid glycoprotein OS=Gallus gallus PE=2 SV=1
156	Q766V2	19	6	Zonapellucida protein D OS=Gallus gallus PE=2 SV=2
160	Q5G8Y9	36	6	Apolipoprotein D OS=Gallus gallus PE=2 SV=1
161	F1NSC7	65	5	Uncharacterized protein OS=Gallus gallus PE=4 SV=3
162	A0A140T8F5	8	5	Uncharacterized protein OS=Gallus gallus PE=4 SV=1
163	E1BZN8	14	6	Uncharacterized protein OS=Gallus gallus PE=3 SV=2
164	Q6IV20	62	11	Gallinacin-11 OS=Gallus gallus PE=1 SV=1
165	A0A1D5PK48	59	9	Uncharacterized protein OS=Gallus gallus PE=4 SV=1
167	A0A1D5PEY8	26	5	Uncharacterized protein OS=Gallus gallus PE=4 SV=1
168	A0A1L1RQF3	64	6	Uncharacterized protein OS=Gallus gallus PE=4 SV=1
172	A0A1D5PLZ2	19	6	Uncharacterized protein OS=Gallus gallus PE=3 SV=1
173	P68139	19	1	Actin alpha skeletal muscle OS=Gallus gallus PE=1 SV=1
177	A0A1L1RNR4	12	5	Uncharacterized protein OS=Gallus gallus PE=4 SV=1
178	A0A1L1RSQ4	46	5	Uncharacterized protein OS=Gallus gallus PE=4 SV=1
181	Q25C36	16	5	Olfactomedin-like protein 3 OS=Gallus gallus PE=2 SV=1
182	F1NL38	10	4	Uncharacterized protein OS=Gallus gallus PE=4 SV=3

Cont. Table 1.

Table 1., Cont. ...

Protein ID	Uni Prot Entry	Coverage (%)	Unique	Protein description
183	A0A1D5PV72	34	3	Uncharacterized protein OS=Gallus gallus PE=4 SV=1
186	F1NJU5	9	4	Uncharacterized protein OS=Gallus gallus PE=4 SV=2
194	A0A1D5PV66	24	1	Uncharacterized protein OS=Gallus gallus PE=4 SV=1
200	E1BTQ4	23	4	Uncharacterized protein OS=Gallus gallus PE=4 SV=1
201	A0A1D5PZU8	36	2	Uncharacterized protein OS=Gallus gallus PE=4 SV=1
202	F1NPJ8	23	4	Glutathione peroxidase OS=Gallus gallus PE=3 SV=2
203	F1NWP1	10	5	Coagulation factor IX OS=Gallus gallus PE=3 SV=1
208	A0A0K0PUH6	29	4	Chemerin OS=Gallus gallus PE=2 SV=1
210	E1C7C1	8	4	Uncharacterized protein OS=Gallus gallus PE=4 SV=2
211	P25155	15	5	Coagulation factor X OS=Gallus gallus PE=1 SV=1
212	A0A1D5PM82	38	1	Uncharacterized protein OS=Gallus gallus PE=4 SV=1
216	A0A1D5P238	6	4	Uncharacterized protein OS=Gallus gallus PE=4 SV=1
218	F1NF64	10	6	Uncharacterized protein OS=Gallus gallus PE=3 SV=3
222	F1NI04	8	3	N-acetylglucosamine-6-sulfatase OS= Gallus gallus PE=3 SV=2
223	A0A1D5PJ81	29	2	Uncharacterized protein OS=Gallus gallus PE=4 SV=1
224	E1BZE1	14	4	Uncharacterized protein OS=Gallus gallus PE=3 SV=1
225	O93601	10	3	Apolipoprotein AIV OS=Gallus gallus PE=2 SV=1
226	F1NEB3	10	3	Uncharacterized protein OS=Gallus gallus PE=3 SV=2
227	F1NV36	5	5	Uncharacterized protein OS=Gallus gallus PE=4 SV=2
228	P01038	38	4	Cystatin OS=Gallus gallus PE=1 SV=2
229	Q90773	11	3	Protein CEPU-1 OS=Gallus gallus PE=1 SV=1
236	F1NC22	46	3	Uncharacterized protein OS=Gallus gallus PE=4 SV=4

Cont. Table 1.

Table 1., Cont. ...

Protein ID	Uni Prot Entry	Coverage (%)	Unique	Protein description
238	A0A1D5PU00	9	3	Uncharacterized protein OS=Gallus gallus PE=4 SV=1
241	F1N9N4	34	3	Uncharacterized protein OS=Gallus gallus PE=4 SV=1
243	D5GR58	51	4	Gallin protein OS=Gallus gallus PE=4 SV=1
246	F1NSC8	47	1	Uncharacterized protein OS=Gallus gallus PE=4 SV=4
248	A0A1D5PAQ0	23	1	Uncharacterized protein OS=Gallus gallus PE=4 SV=1
249	A0A1D5P1L5	25	3	Uncharacterized protein OS=Gallus gallus PE=4 SV=1
250	F1NSD3	47	3	Uncharacterized protein OS=Gallus gallus PE=4 SV=3
257	Q90839	13	3	Dickkopf-related protein 3 OS=Gallus gallus PE=2 SV=1
262	F1NYK2	8	4	Sulfhydryl oxidase OS=Gallus gallus PE=4 SV=3
270	F1NYT7	6	3	Uncharacterized protein OS=Gallus gallus PE=3 SV=3
276	E1BS40	7	3	Uncharacterized protein OS=Gallus gallus PE=4 SV=3
291	P32760	35	3	Pleiotrophin OS=Gallus gallus PE=1 SV=1
304	F1NIN2	6	2	Carboxypeptidase OS=Gallus gallus PE=3 SV=2
308	E1C6L4	4	2	Uncharacterized protein OS=Gallus gallus PE=4 SV=1
309	O57391	6	2	Gamma-enolase OS=Gallus gallus PE=2 SV=1
312	F1P181	7	2	Uncharacterized protein OS=Gallus gallus PE=4 SV=1
320	Q710A0	12	2	Secreted phosphoprotein 24 OS=Gallus gallus PE=2 SV=1
324	F1NPN3	4	2	Uncharacterized protein OS=Gallus gallus PE=3 SV=3
325	P81476	23	2	Ribonuclease CL2 OS=Gallus gallus PE=1 SV=1
327	E1C7H6	5	2	Uncharacterized protein OS=Gallus gallus PE=3 SV=2
335	A0A1D5PSQ1	2	1	Uncharacterized protein OS=Gallus gallus PE=4 SV=1
337	A0A1D5P3J3	5	1	Uncharacterized protein OS=Gallus gallus PE=4 SV=1
340	A0A1D5PQ92	15	1	Uncharacterized protein OS=Gallus gallus PE=3 SV=1

Cont. Table 1.

Table 1., Cont. ...

Protein ID	Uni Prot Entry	Coverage (%)	Unique	Protein description
349	P00356	7	2	Glyceraldehyde-3-phosphate dehydrogenase OS=Gallus gallus PE=2 SV=3
351	A0A1D5PUA3	0	2	Low-density lipoprotein receptor-related protein 1 OS=Gallus gallus PE=4 SV=1
352	F1NLP7	4	2	Antithrombin-III OS=Gallus gallus PE=3 SV=2
358	A0A1L1RPK6	7	1	Uncharacterized protein OS=Gallus gallus PE=4 SV=1
359	Q9DDD4	17	2	Tetranectin OS=Gallus gallus PE=2 SV=1
371	F1NBI4	2	2	Uncharacterized protein OS=Gallus gallus PE=4 SV=2
373	F1NNV4	1	2	Peptidylprolylisomerase OS=Gallus gallus PE=4 SV=3
376	Q5ZMK2	1	1	Uncharacterized protein OS=Gallus gallus PE=2 SV=1
380	A0A1D5P366	0	1	Uncharacterized protein OS=Gallus gallus PE=4 SV=1
381	E1BR42	1	2	Uncharacterized protein OS=Gallus gallus PE=3 SV=2
388	Q8AV77	34	5	Hep21 protein OS=Gallus gallus PE=2 SV=1
392	A0A1D5PYI5	10	1	Uncharacterized protein OS=Gallus gallus PE=4 SV=1
396	A0A1D5PAG9	2	1	Uncharacterized protein OS=Gallus gallus PE=4 SV=2
400	A0A1D5NVV9	1	1	Uncharacterized protein OS=Gallus gallus PE=4 SV=1
402	F1NHI4	5	1	Superoxide dismutase [Cu-Zn] OS=Gallus gallus PE=3 SV=3
406	A0A1D5NUV4	1	1	Uncharacterized protein OS=Gallus gallus PE=4 SV=1
408	E1C4G2	0	1	Uncharacterized protein OS=Gallus gallus PE=3 SV=2
410	E1C4C8	2	2	Uncharacterized protein OS=Gallus gallus PE=4 SV=4
413	A0A1D5P5L4	3	1	Uncharacterized protein OS=Gallus gallus PE=4 SV=1
422	E1C5Z3	0	1	Uncharacterized protein OS=Gallus gallus PE=4 SV=2
431	P11533	0	1	Dystrophin OS=Gallus gallus PE=2 SV=1
447	A0A1D5P4P1	3	2	Uncharacterized protein OS=Gallus gallus PE=3 SV=1

Cont. Table 1.

Table 1., Cont. ...

Protein ID	Uni Prot Entry	Coverage (%)	Unique	Protein description
457	F1NAR5	5	2	Uncharacterized protein OS=Gallus gallus PE=3 SV=2
459	F1NV00	1	1	Uncharacterized protein OS=Gallus gallus PE=4 SV=3
463	F1NMU1	0	1	Uncharacterized protein OS=Gallus gallus PE=4 SV=4
468	Q2LK96	10	2	Lung lectin OS=Gallus gallus PE=2 SV=1
470	F1P1U0	1	1	Uncharacterized protein OS=Gallus gallus PE=4 SV=3
476	R4GFD0	1	1	Uncharacterized protein OS=Gallus gallus PE=4 SV=2
477	F1NHT5	3	1	Uncharacterized protein OS=Gallus gallus PE=4 SV=2
481	A0A1D5P8P5	3	1	Uncharacterized protein OS=Gallus gallus PE=4 SV=1
488	F1NAS4	1	1	Uncharacterized protein OS=Gallus gallus PE=4 SV=3
490	A0A1L1RXR0	2	1	Uncharacterized protein OS=Gallus gallus PE=4 SV=1
501	Q90986	16	1	Lymphocyte antigen 6E OS=Gallus gallus PE=2 SV=1
512	F1N8T2	1	1	Uncharacterized protein OS=Gallus gallus PE=3 SV=2
515	A0A1D5P1D5	1	1	Uncharacterized protein OS=Gallus gallus PE=4 SV=1
525	A0A1D5NW92	1	1	Uncharacterized protein OS=Gallus gallus PE=4 SV=1
528	A0A1D5PMA3	1	1	Uncharacterized protein OS=Gallus gallus PE=4 SV=1
534	E1C182	1	1	Uncharacterized protein OS=Gallus gallus PE=4 SV=3
547	A0A1D5PIZ8	1	1	Uncharacterized protein OS=Gallus gallus PE=4 SV=1
559	A0A1D5NU22	1	1	Uncharacterized protein OS=Gallus gallus PE=4 SV=1
564	A0A1L1RXE1	5	1	Uncharacterized protein OS=Gallus gallus PE=4 SV=1
568	E1C040	0	1	Uncharacterized protein OS=Gallus gallus PE=4 SV=3
576	A0A1D5PSC1	0	1	Uncharacterized protein OS=Gallus gallus PE=4 SV=1

Cont. Table 1.

Table 1., Cont. ...

Protein ID	Uni Prot Entry	Coverage (%)	Unique	Protein description
584	A0A1D5P3V0	1	1	Histone-lysine N-methyltransferase H3 lysine-79 specific OS=Gallus gallus PE=3 SV=1
585	A0A1D5NXZ3	0	1	Uncharacterized protein OS=Gallus gallus PE=4 SV=1
588	A0A1D5PEA7	6	1	Uncharacterized protein OS=Gallus gallus PE=4 SV=2
592	Q6QLR1	15	1	Gallinacin-9 OS=Gallus gallus PE=2 SV=1
596	E1C2P9	1	1	Kinectin OS=Gallus gallus PE=4 SV=3
599	A0A1D5PHA4	2	1	Uncharacterized protein OS=Gallus gallus PE=4 SV=1
600	A0A1D5P2P4	0	1	Uncharacterized protein OS=Gallus gallus PE=4 SV=1
609	P81021	0	1	Vigilin OS=Gallus gallus PE=2 SV=1
611	A0A1D5PHU2	1	1	Syntaxin-binding protein 1 OS=Gallus gallus PE=3 SV=1
617	A0A1D5PYC5	0	1	Uncharacterized protein OS=Gallus gallus PE=4 SV=1
627	E1BYQ7	0	1	Uncharacterized protein OS=Gallus gallus PE=4 SV=4
634	F1NRP8	0	1	Uncharacterized protein OS=Gallus gallus PE=4 SV=3
635	A0A1D5P5H9	0	1	Uncharacterized protein OS=Gallus gallus PE=4 SV=1
643	A0A1D5P1T9	2	1	Uncharacterized protein OS=Gallus gallus PE=4 SV=2
649	A0A1D5P7U6	3	1	Uncharacterized protein OS=Gallus gallus PE=4 SV=1
653	A0A1L1RJ89	3	1	Uncharacterized protein OS=Gallus gallus PE=3 SV=1
664	A0A1D5PXD7	0	1	Uncharacterized protein OS=Gallus gallus PE=4 SV=1
683	A0A1D5PCF7	0	1	Uncharacterized protein OS=Gallus gallus PE=4 SV=1
694	A0A1D5PVY0	1	1	Uncharacterized protein OS=Gallus gallus PE=4 SV=1
698	E1C796	0	1	Uncharacterized protein OS=Gallus gallus PE=3 SV=3
699	R4GJS2	1	1	Uncharacterized protein OS=Gallus gallus PE=4 SV=2
702	A0A1D5PIW9	0	1	Uncharacterized protein OS=Gallus gallus PE=4 SV=1

Cont. Table 1.

Table 1., Cont. ...

Protein ID	Uni Prot Entry	Coverage (%)	Unique	Protein description
704	Q5ZKI4	1	1	Coiled-coil domain-containing protein 93 OS=Gallus gallus PE=2 SV=1
707	A0A1L1RPA7	6	1	Uncharacterized protein OS=Gallus gallus PE=4 SV=1
708	A0A1L1RJY4	4	1	Uncharacterized protein OS=Gallus gallus PE=4 SV=1
724	A0A1D5PHS1	2	1	Phospholipid-transporting ATPase OS= Gallus gallus PE=3 SV=1
737	A0A1D5P0K2	1	1	Uncharacterized protein OS=Gallus gallus PE=4 SV=1
739	A0A1D5P3U7	1	1	Voltage-dependent R-type calcium channel subunit alpha OS=Gallus gallus PE=3 SV=1
740	F1NVI1	3	1	Uncharacterized protein OS=Gallus gallus PE=4 SV=2
748	E1BS56	2	1	Uncharacterized protein OS=Gallus gallus PE=3 SV=1
762	A0A1D5P4W7	2	2	Uncharacterized protein OS=Gallus gallus PE=4 SV=1
763	F1NHT3	1	1	Spectrin alpha chain non-erythrocytic 1 OS=Gallus gallus PE=4 SV=2
767	A0A1D5NY32	1	1	Uncharacterized protein OS=Gallus gallus PE=4 SV=2
770	A0A1D5PEK5	1	1	Uncharacterized protein OS=Gallus gallus PE=4 SV=1
775	R4GKI4	4	1	Gamma-glutamyl cyclo transferase OS= Gallus gallus PE=3 SV=1
777	A0A1D5NYJ6	1	1	Uncharacterized protein OS=Gallus gallus PE=4 SV=2
783	E1BT78	1	1	Uncharacterized protein OS=Gallus gallus PE=4 SV=1
798	A0A1D5PUS7	1	1	Uncharacterized protein OS=Gallus gallus PE=4 SV=1
805	F1P300	1	1	Transporter OS=Gallus gallus PE=3 SV=3
807	F1P088	1	1	Uncharacterized protein OS=Gallus gallus PE=4 SV=4
808	A0A1D5PDJ1	1	1	ATP-dependent 6-phosphofructokinase OS=Gallus gallus PE=3 SV=1
816	E1BQP9	1	1	Uncharacterized protein OS=Gallus gallus PE=4 SV=3
820	E1C8N4	1	1	Uncharacterized protein OS=Gallus gallus PE=4 SV=3

Cont. Table 1.

Table 1., Cont. ...

Protein ID	Uni Prot Entry	Coverage (%)	Unique	Protein description
828	F1P040	1	1	Uncharacterized protein OS=Gallus gallus PE=3 SV=1
837	A0A1D5PKV8	1	1	Uncharacterized protein OS=Gallus gallus PE=4 SV=1
840	A0A1D5PUJ3	1	1	Uncharacterized protein OS=Gallus gallus PE=4 SV=1
844	E1BWI0	0	1	Uncharacterized protein OS=Gallus gallus PE=4 SV=4
847	F1NA61	2	2	Uncharacterized protein OS=Gallus gallus PE=4 SV=1
848	A0A1D5NU51	2	1	Uncharacterized protein OS=Gallus gallus PE=4 SV=1
860	A0A1D5PJM2	1	1	Uncharacterized protein OS=Gallus gallus PE=4 SV=1
866	A0A1D5PAW6	0	1	Uncharacterized protein OS=Gallus gallus PE=3 SV=1
869	F1P5N3	2	1	Uncharacterized protein OS=Gallus gallus PE=4 SV=3
874	F1NVF5	2	1	Ubiquitin carboxyl-terminal hydrolase BAP1 OS=Gallus gallus PE=4 SV=2
880	A0A1D5NZW8	0	1	Uncharacterized protein OS=Gallus gallus PE=4 SV=1
903	F1NJT3	0	1	Fibronectin OS=Gallus gallus PE=4 SV=1
907	A0A1D5PZV3	1	1	Uncharacterized protein OS=Gallus gallus PE=4 SV=1
916	A0A1D5NXE0	1	1	Uncharacterized protein OS=Gallus gallus PE=4 SV=2
923	F1NIK1	2	1	Transmembrane channel-like protein OS= Gallus gallus PE=3 SV=3
933	A0A1L1RQG0	5	1	Uncharacterized protein OS=Gallus gallus PE=4 SV=1
939	R9PXN0	5	1	Swi5-dependent recombination DNA repair protein 1 homolog OS=Gallus gallus PE=4 SV=2
944	A0A1D5NVB3	3	1	Uncharacterized protein OS=Gallus gallus PE=4 SV=1
946	A0A1D5PZL3	1	1	Uncharacterized protein OS=Gallus gallus PE=4 SV=1
953	E1BVK3	2	1	Uncharacterized protein OS=Gallus gallus PE=4 SV=3
976	E1BTU4	5	1	Uncharacterized protein OS=Gallus gallus PE=4 SV=4

Cont. Table 1.

Table 1., Cont. ...

Protein ID	Uni Prot Entry	Coverage (%)	Unique	Protein description
990	F1NQ85	2	1	Uncharacterized protein OS=Gallus gallus PE=4 SV=2
999	A0A1D5PR51	2	1	Uncharacterized protein OS=Gallus gallus PE=3 SV=1
1002	A0A1D5NUC3	1	1	Uncharacterized protein OS=Gallus gallus PE=4 SV=1
1007	A0A1D5PUV1	0	1	Uncharacterized protein OS=Gallus gallus PE=4 SV=1
1013	A0A1D5PYP1	1	1	Uncharacterized protein OS=Gallus gallus PE=4 SV=1
1017	O73590	0	1	Zinc finger homeobox protein 4 OS= Gallus gallus PE=2 SV=2
1024	A0A1D5PME0	3	1	Uncharacterized protein OS=Gallus gallus PE=4 SV=1
1025	A0A1D5PVL6	1	1	Uncharacterized protein OS=Gallus gallus PE=4 SV=1
1026	A0A1D5PRU1	4	1	Uncharacterized protein OS=Gallus gallus PE=4 SV=1
1032	A0A140T8F7	3	1	Beta-nerve growth factor OS=Gallus gallus PE=3 SV=2
1034	E1BRD0	3	1	Uncharacterized protein OS=Gallus gallus PE=4 SV=1
1043	E1C224	0	1	Uncharacterized protein OS=Gallus gallus PE=4 SV=4
1047	E1BUV5	1	1	Uncharacterized protein OS=Gallus gallus PE=4 SV=4
1101	Q90XB3	2	1	Oligodendrocyte transcription factor 2 OS=Gallus gallus PE=2 SV=1
1114	A0A1D5PDY3	1	1	Uncharacterized protein OS=Gallus gallus PE=3 SV=1
1115	A0A1L1RJ80	1	1	Uncharacterized protein OS=Gallus gallus PE=4 SV=1
1124	A0A1D5PQH9	0	1	Uncharacterized protein OS=Gallus gallus PE=4 SV=1
1144	F1NS33	4	1	Uncharacterized protein OS=Gallus gallus PE=4 SV=3
1167	A0A1D5PFY6	2	1	Uncharacterized protein OS=Gallus gallus PE=4 SV=1
1187	F1NSW3	1	1	Uncharacterized protein OS=Gallus gallus PE=4 SV=3
1188	F1N916	10	1	Uncharacterized protein OS=Gallus gallus PE=3 SV=2

Cont. Table 1.

Table 1., Cont. ...

Protein ID	Uni Prot Entry	Coverage (%)	Unique	Protein description
1201	E1C8S8	2	1	Uncharacterized protein OS=Gallus gallus PE=3 SV=1
1256	A0A1L1RQD1	6	1	Protein RER1 OS=Gallus gallus PE=4 SV=1
1257	F1P3T8	1	1	Uncharacterized protein OS=Gallus gallus PE=3 SV=3
1259	E1C5Z0	1	1	Uncharacterized protein OS=Gallus gallus PE=4 SV=4
1298	F1NGJ3	0	1	Uncharacterized protein OS=Gallus gallus PE=4 SV=2
1300	P11029	0	1	Acetyl-CoA carboxylase OS=Gallus gallus PE=1 SV=1
1320	E1C7U0	2	1	Stimulator of interferon genes protein OS=Gallus gallus PE=3 SV=1
1323	A0A1D5NW84	1	1	Uncharacterized protein OS=Gallus gallus PE=3 SV=1
1391	A0A1D5NVW9	3	1	Uncharacterized protein OS=Gallus gallus PE=4 SV=1
1413	A0A1D5PUC6	3	1	Uncharacterized protein OS=Gallus gallus PE=4 SV=1
1424	Q6QLQ9	16	1	Gallinacin-10 OS=Gallus gallus PE=2 SV=1
1474	A0A1D5PR82	4	1	Uncharacterized protein OS=Gallus gallus PE=4 SV=1
1482	F1NW34	2	1	Uncharacterized protein OS=Gallus gallus PE=4 SV=3
1505	E1BR23	2	1	Multidrug and toxin extrusion protein OS=Gallus gallus PE=3 SV=2
1531	Q5ZHV8	1	1	RecQ-mediated genome instability protein 1 OS=Gallus gallus PE=2 SV=1
1729	A0A1D5PHW5	1	1	Uncharacterized protein OS=Gallus gallus PE=4 SV=1
1738	A0A1D5PB41	1	1	Uncharacterized protein OS=Gallus gallus PE=4 SV=1
1761	Q6RW63	1	1	Neurofibromatosis 2 OS=Gallus gallus PE=2 SV=1
2013	F1P5G6	2	1	Uncharacterized protein OS=Gallus gallus PE=4 SV=3
2121	F1NPT1	1	1	Uncharacterized protein OS=Gallus gallus PE=4 SV=2
2342	A0A1D5NV50	4	1	Carboxylic ester hydrolase OS=Gallus gallus PE=3 SV=1
2407	R4GIL1	4	1	Paired mesoderm homeobox protein 2 OS=Gallus gallus PE=3 SV=1

Cont. Table 1.

Table 1., Cont. ...

Protein ID	Uni Prot Entry	Coverage (%)	Unique	Protein description
2409	F1NFW9	9	1	Uncharacterized protein OS=Gallus gallus PE=3 SV=3
2453	Q2UXM6	2	1	RNase L inhibitor OS=Gallus gallus PE=2 SV=1
2724	A0A1L1RRL6	2	1	Uncharacterized protein OS=Gallus gallus PE=4 SV=1
3415	A0A1D5P9J0	1	1	Uncharacterized protein OS=Gallus gallus PE=4 SV=1

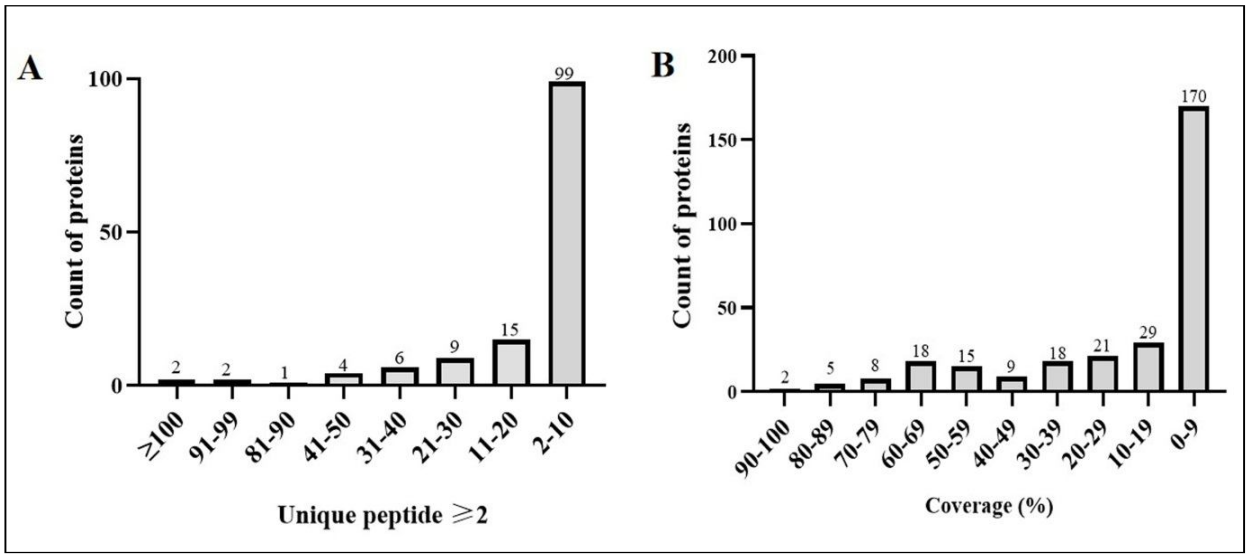

Fig. 1. Basic information and statistics of FYEY proteome LC-MS/MS analysis. (A) Count of proteins in different coverage rates. (B) Count of proteins in different unique peptides

hormone mainly play a role in this process. Molecular functional ontology analysis was as follows: the most abundant proteins were protein binding (43), ATP binding (19), calcium ion binding (17), serine endopeptidase inhibitor activity (16), metal ion binding (14), and serine-type endopeptidase activity (13). In addition, there were also 7 proteins involved in lipid transport, which were basically consistent with those involved in lipid transport in biological processes. The most abundant cell components include extracellular space (43), extracellular vesicles (42), extracellular region (33), membrane composition (30), nucleus (17), and cytoplasm (17), indicating that the proteins contained in FEYE are basically secretory proteins.

KEGG pathway analysis: Based on KEGG analysis, the top 20 pathways most enriched are shown in Fig. 3. The first 10 pathways were Regulation of actin cytoskeleton (gga04810), Metabolic pathway (gga01100), Focal adhesion (gga04510), Endocytosis (gga04144) and ECM-receptor interaction (gga04512), Glycolysis/ Gluconeogenesis (gga00010), Carbon metabolism (gga01200), Biosynthesis of amino acids (gga01230), PPAR signaling pathway (gga03320), MAPK signaling pathway (gga04010). Regulation of actin cytoskeleton is the most abundant, involving 8 proteins, suggesting that FEYE can regulate the formation of cytoskeleton. The proteins involved in each pathway are detailed in Table 2.

Table 2. Proteins contained in different pathways in KEGG

Pathway name	Pathway ID	Protein name	Count
Regulation of actin cytoskeleton	gga04810	F2, GSN, GNS (isoform X2), ACTG1, FN1, ARHGEF12, ITGA2, IQGAP1	8
Metabolic pathways	gga01100	GNS, GAPDH, ENO2, PFKL, CEL, ACAC	6
Focal adhesion	gga04510	VTN, ACTG1, FN1, ITGA2, MET, COL4A6	6
Endocytosis	gga04144	RBP, VPS37A, MET, GBF1	4
ECM-receptor interaction	gga04512	VTN, FN1, ITGA2, COL4A6	4
Glycolysis/ Gluconeogenesis	gga00010	GAPDH, ENO2, PFKL	3
Carbon metabolism	gga01200	GAPDH, ENO2, PFKL	3
Biosynthesis of amino acids	gga01230	GAPDH, ENO2, PFKL	3
PPAR signaling pathway	gga03320	APOA1, APOC3, PLIN1	3
MAPK signaling pathway	gga04010	NGF, CACNA1E, NRK	3
Lysosome	gga04142	GNS, NPC2, CTSA	3
Phagosome	gga04145	C3, ACTG1, ITGA2	3
Apoptosis	gga04210	ACTG1, SPTAN1, NGF	3
Adherens junction	gga04520	ACTG1, IQGAP1, MET	3
Influenza A	gga05164	PLG, ACTG1, EIF2AK4	3
Herpes simplex infection	gga05168	C3, C5, EIF2AK4	3
Pantothenate and CoA biosynthesis	gga00770	VNN1 (Vascular non-inflammatory molecule 2-like), VNN1 (Pantetheinase-like)	2
Terpenoid backbone biosynthesis	gga00900	PCYOX1, ZMPSTE24	2
RNA degradation	gga03018	ENO2, PFKL	2
Neuroactive ligand-receptor interaction	gga04080	PLG, F2	2

Protein-protein interaction network analysis: To explore lipid metabolism-related proteins in FEYE proteins, we submitted the top 50 proteins to STRING database for protein-protein interactions (Protein protein interaction, PPI) based on abundance rankings (Fig. 4). PPI contains 50 proteins, 4 proteins were associated with lipid development, including APOA1, APOB, APOH, VTG2 (Yellow). However, these 4 proteins do not exist alone; in addition to the interaction between ZP1 and ZP3, each protein is associated and interacts with each other, participates in the development process of FEYE, and provides a basis for subsequent research.

Verification of the results of LC-MS/MS analysis of FEYE: FEYE was first subjected to gradient dilution, and each diluted gradient of FEYE was subjected to SDS-PAGE electrophoresis, and then the gel was stained with Coomassie Brilliant blue G-250. The results showed a significant towing in the display 10^{-1}, the 10^{-2} protein band was the most obvious, and the 10^{-3}, 10^{-4}, 10^{-5} protein bands were not obvious. Thus, we chose a protein of 10^{-2} for the next experiment (Fig. 5A).

Based on the results of KEGG pathway, we found that CEL (Bile salt-activated lipase) in the metabolic pathway and APOA1 in the PPAR

Active ingredients analysis in fertilized hen egg extract

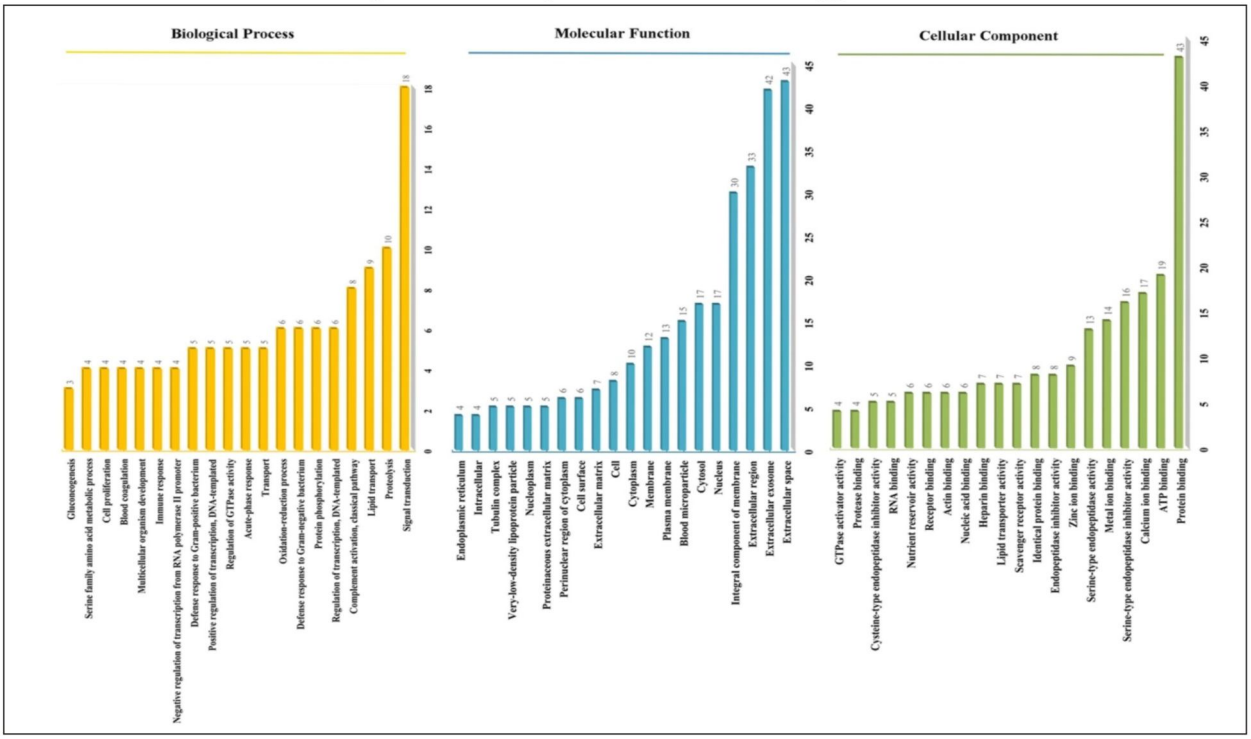

Fig. 2. GO classification results can be visualized using Bar graphs. The GO classification Bar map plots the top 20 pathways with the highest number of proteins. The ordinate indicates the number of total proteins in each secondary classification. The color indicates different primary classifications. Biological Process (Yellow), Molecular Function (Blue), Cellular Component (Green)

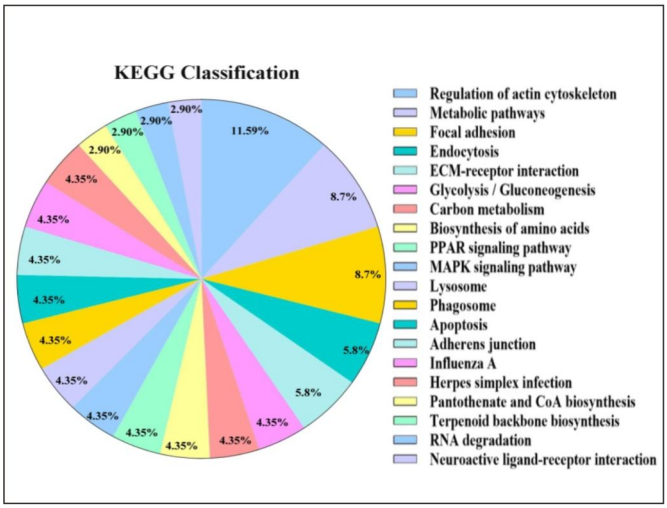

Fig. 3. The KEGG pathway annotation results are visualized using the pie Chart. The KEGG classification Bar plots the top 20 pathways with the highest number of proteins, and the ordinate indicates the percentage of protein in the total protein

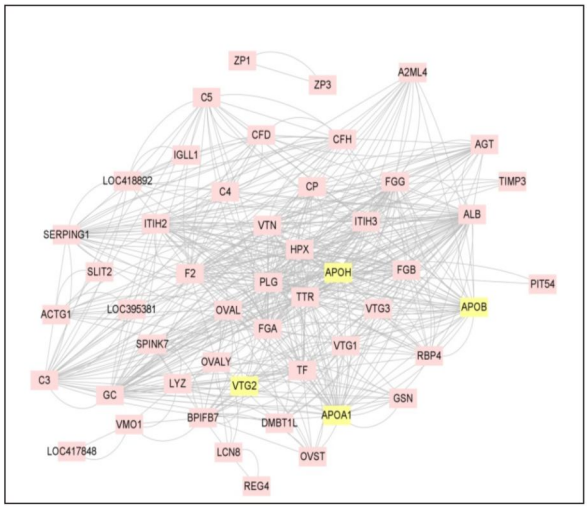

Fig. 4. Protein-protein interaction network analysis of proteins in FEYE

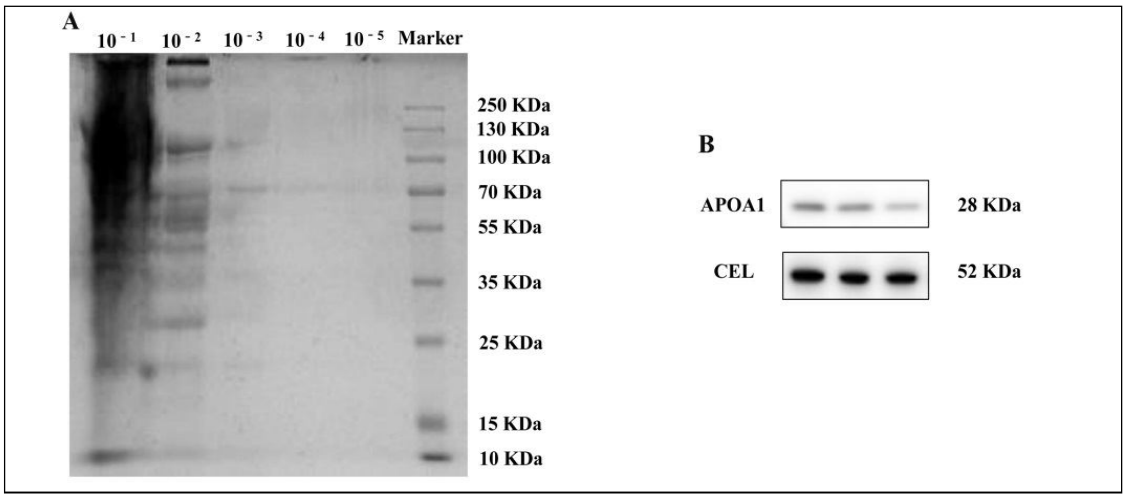

Fig. 5. Verification of FEYE related proteins by western blotting. (A) SDS-PAGE analysis of FEYE. Mr: molecular mass ladder. Samples: Gradient diluted FEYE. Staining with Coomassie blue G50. (B) Protein expression of APOA1 and CEL in FEYE

pathway are involved in lipid metabolism (Table 2). Then western blot was used to verify that APOA1 and CEL were indeed expressed in FEYE (Fig. 5B).

DISCUSSION

Many human diseases are caused by dietary components with high fat and high sugar. Improving dietary components can not only ensure adequate nutrition but also reduce the occurrence of diseases. It has been reported that the nutritional value of fertilized hen eggs is much higher than that of ordinary eggs, and egg yolk is the essence of the whole egg. Its rich protein content can improve the taste of food and has antioxidant functions. In this study, FEYE was analyzed by LC-MS/MS to screen proteins for improving dietary composition.

The distribution of FEYE in GO analysis (Biological process, Bellular component and Molecular function) reflects the biological function of FEYE in different physiological processes. Signal transduction, protein hydrolysis and lipid transport are the most abundant proteins in biological processes. Apolipoprotein family and vitellogenin play a role in lipid transport. According to the analysis of KEGG pathway, the most enriched pathways were the Regulation of actin cytoskeleton, metabolic pathway and Focal adhesion. However, the pathway related to FEYE and lipid metabolism is PPAR pathway, and the proteins involved are APOA1, APOC3 and Perilipin-1; in addition, in the metabolic pathway, the protein involved in lipid transport is CEL. Apolipoprotein is a protein component that constitutes plasma lipoprotein, which is mainly divided into five categories: A, B, C, D and E. The basic function is to carry lipids and stabilize the structure of lipoprotein. Some apolipoproteins also have the functions of activating lipoprotein metabolic enzymes and recognizing receptors. APOB 100 is one of the main expression forms of human apolipoprotein. It is also the main component of very low density lipoprotein (VLDL), low density lipoprotein (LDL) and intermediate density lipoprotein (IDL); it plays an extremely important role in the transport and metabolism of lipids and cholesterol in animals. Ovalbumin is a secreted protein which is the main nutritional ingredient in egg white. However, in this FEYE proteomics identification, it was found that its protein abundance ranked first, which proved that it was also expressed in FEYE and the content was high. As an egg yolk precursor protein, vitellogenin (VTG) is a member of the large lipid transport protein

(LLTP) superfamily. VTG exists in almost all ovipositing females and provides nutrition for developing embryos (Sun *et al.*, 2021). VTG is also proven to be an immune component factor that can protect the host from microbial attacks including bacteria and viruses. In addition, VTG also has antioxidant activity, which can protect cells from free radical damage. VTG2, as a subtype of VTG, is first in protein abundance in this proteomics analysis, and it has been confirmed in our research group that it can promote muscle development and stimulate the browning of white fat (Li *et al.*, 2021). In summary, it suggests that FEYE can be used as a raw material for food to play a role in nutrition, immunity, antioxidant, and fat browning.

Adipose tissue is the largest energy pool in mammals, which is considered to be a dynamic organ with complex decomposition and synthesis metabolism. There are mainly two types, white adipose tissue (WAT) and brown adipose tissue (BAT). White adipose tissue is the main part of fat storage, while brown adipose tissue has the ability to produce heat without shaking. Adipose tissue also secretes a variety of important endocrine hormones, such as leptin, adiponectin and resistin, so regulating lipid metabolism is very important in life. Giaginis *et al*. (2008) found that PPARγ plays a key role in placenta biology (Giaginis *et al.*, 2008). The decrease of PPARγ in the inguinal region fat nucleus receptor of aging mice can increase the expansion of adipose tissue and insulin resistance. These metabolic effects are associated with decreased heat production, decreased brown fat gene level, and browning of subcutaneous adipose tissue (Xu *et al.*, 2018). Activation of PPARα leads to cellular fatty acid absorption and lipoprotein metabolism, thereby reducing triglyceride levels and increasing high density lipoprotein cholesterol levels (Ferré, 2004). The results of this study showed that three proteins, APOA1, APOC3 and Perilipin-1, were detected in PPAR pathway. APOA1 is the main protein component of high density lipoprotein (HDL) in plasma and is the key component in regulating lipoprotein metabolism and cardiovascular disease risk (Lai *et al.*, 2005). It is also a trigger for non-trembling brown fat that generates heat (Kalogeropoulou *et al.*, 2020). APOC3 is mainly produced in the liver, which is a regulator of triglyceride (TG) and has the function of inhibiting lipoprotein lipase and hepatic lipase activity (Rodríguez *et al.*, 2022). Perilipin-1 is located on the lipid droplet surface of adipocytes and regulates the storage and hydrolysis of triglycerides. When obese, the level of perilipin in adipocytes decreases, resulting in a decrease in fat decomposition rate. Compared with wild-type mice, the white fat and body weight of Perilipin transgenic mice decreased, indicating that increasing the expression of the Perilipin gene can prevent diet-induced obesity (Bae *et al.*, 2017). CEL is a multifunctional lipase (Wang and Hartsuck, 1993). In early studies, CEL was added to a milk-based diet, which could activate lipase and promote fat digestion, thereby increasing the growth rate (Tang and Wang, 1990). Studies have also found that CEL can improve pancreatic dysfunction and poor fat absorption caused by pathological causes (Wang *et al.*, 2017).

In summary, LC-MS/MS analysis of FEYE protein can provide a solid theoretical basis for subsequent research on fertilized hen eggs. After WB verification, we found that APOA1 and CEL related to lipid metabolism were indeed expressed in FEYE. Therefore, it is suggested that there are proteins regulating lipid metabolism in fertilized hen eggs, which provides a new idea for the change of dietary function.

Conflict of interest: The authors declare no conflicts of interest related to this study.

Author contributions: PM, YL, YG, LX: Analyzed the data; XY, YY: Made the figures; WH, JL: Provided technical assistance; XL: Revised the paper; HW: Designed the experiments and provided funds. All authors read and approved the final manuscript.

Ethics approval and consent to participate: All animal operations were carried out in

accordance with the "Guidelines for the Care and Use of Laboratory Animals of Shanxi Agricultural University" and were approved by the Animal Medicine Committee of Shanxi Agricultural University. participates in the development process of FEYE, and provides a basis for subsequent research.

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