Effect of different diets on the hepatopancreatic proteomes of Chinese mitten crab, Eriocheir sinensis

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Research

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Abstract

Background: Aquatic plants and freshwater snails are important natural food sources of *Eriocheir sinensis*, which play important roles in the growth and development of *E. sinensis*. However, research on how aquatic plants and freshwater snails affect the growth and development of *E. sinensis* remains scant.

Methods: The effects of the two kinds of natural food sources on the growth and development of *E. sinensis* were studied by determining the hepatopancreatic proteomes of three groups, namely, *E. sinensis* fed with aquatic plants combined with freshwater snails (group A), *E. sinensis* fed with aquatic plants only (group B), and *E. sinensis* fed with freshwater snails only (group C), using tandem mass tag technology.

Results: A total of 110 differentially expressed proteins between groups A and B were identified, among which 78 were up-regulated and 32 were down-regulated in group A. Meanwhile, nine proteins were up-regulated, and 14 proteins were down-regulated in group A relative to those in group C. The proteins related to molting and growth such as cryptocyanin and cuticle protein CBM were up-regulated in group A compared with group B. The immunity-related proteins, such as mannosyl-oligosaccharide glucosidase and glutathione peroxidase, that were differentially expressed between groups A and C, were up-regulated in group A.

Conclusion: The results indicated that freshwater snails might promote the growth and development of *E. sinensis* to a certain extent, and aquatic plants might play an important role in the immunity of *E. sinensis.* Our study provides a theoretical basis for the practice of “providing aquatic plants and freshwater snails” in the ecological culture of *E. sinensis.*

Background

Chinese mitten crab (*Eriocheir sinensis*), is an important economic aquaculture species in China (1). The planting of aquatic plants has become one of the key factors for the success of *E. sinensis* culture. Aquatic plants can not only regulate the pH of water and provide shelter to *E. sinensis*, but also they can be used as food sources by *E. sinensis* due to their rich nutrient contents (2, 3). Researchers have indicated that aquatic plants are beneficial for the growth of *E. sinensis* and can improve the nutritional quality of the edible parts of *E. sinensis*. *E. sinensis* ingest a certain number of aquatic plants to meet their nutritional needs even when receiving sufficient feed (4). Freshwater snails are a high-quality natural feed for *E. sinensis*, and snail feeding in the aquaculture practice can increase the yield and improve the quality of *E. sinensis*(5, 6). Moreover, snails, as an animal-type feed, can increase the content of animal protein in the daily diet, which has a positive influence on the growth of *E. sinensis* (7).

The growth and development of organisms are closely related to the daily food sources. Different food sources cause dramatic changes in the composition of functional proteins in tissues and organs, such as the digestive and metabolic organs; affect biological processes, such as digestion and absorption, energy metabolism, and immune response; and further affect the growth and development of organisms (8, 9). Comparing the liver proteomes of rats fed with animal protein with those of rats fed with plant protein revealed the two groups exhibited drastic changes in their protein expression profiles and considerably

difference on amino acid and fatty acid metabolism (10). The content of proteins related to lipid, carbohydrate, and amino acid metabolism changed in the livers of *Oreochromis niloticus* fed with daily diets containing different nutrients, and the immune systems of the experimental organisms was also affected (11). Replacing dietary fish oil with linseed oil, resulted in considerable changes in the protein expression profile in the hepatopancreas of *E. sinensis*, and subsequently, the capability of *E. sinensis* to adapt to the environment was also altered (12). Food sources can obviously affect the protein composition of organisms and then further affect the growth and development of organisms. Aquatic plants and freshwater snails, as two kinds of important natural food, play important roles in the growth and development of *E. sinensis*. However, research on how aquatic plants and snails affect the growth and development of *E. sinensis* remains scant.

A comprehensive analysis of the composition and dynamics of functional proteins offers important insights into the roles of aquatic plants and snails in the aquaculture practice of *E. sinensis*. Therefore, in this study, three diverse feed types were provided as daily diets for *E. sinensis*: freshwater snail (*Sinotaia quadrata*); waterweed plants (*Elodea canadensis*); and a mixed diet of *S. quadrata* and *E. canadensis*. Then, the protein profiles of *E. sinensis* under the three different feed types were determined and compared to investigate the effect of aquatic plants and snails on the growth and development of *E. sinensis*.

Methods

Sample collection and ethics statement

Juvenile *E. sinensis* (approximately 7.5 g) with the same growth conditions were collected from the Aquatic Animal Germplasm Resource Station of Shanghai Ocean University (Shanghai, China). They were cultured in a circulating water system for seven days to adapt to the environment. Then, the *E. sinensis* individuals were randomly divided into three groups and fed as follows: Group A was fed with a mixed diet of *S. quadrita* and *E. canadensis*, group B was fed with *E. canadensis* only, and group C was fed with *S. quadrita* only. All the *E. sinensis* individuals were reared in the circulating water system, named “Crab Dragon Palace” to maintain consistent culture environment. The water temperature was maintained at 26 ℃ ± 2 ℃, and the three groups were fed with abundant food at 9:00 and 16:00 every day, respectively. When the *E. sinensis* grew to the prestage of molting, their hepatopancreas tissues were collected. Three biological replicateswere collected for each group. Then, the tissues were quickly frozen in liquid nitrogen and stored in a −80 ℃ refrigerator. The study was approved by the Institutional Animal Care and Use Committee of Shanghai Ocean University (Shanghai, China). Sampling procedures complied with the guidelines of the Institutional Animal Care and Use Committee on the care and use of animals for scientific purposes.

Protein extraction and quality control

The collected hepatopancreatic tissues were taken from −80 °C refrigerator and homogenized. Approximately 50 mg of minced tissue was mixed with 500 μl of RIPA lysate (PMSF was added before use). Subsequently, the homogenate was incubated on an ice bath for 30 min. Centrifugation was performed at 14 000 g for 10 min at 4 °Cand the supernatant was collected. Protein concentration was measured with a

Pierce BCA protein assay kit in accordance with instructions (Thermo fisher, USA), and protein quality was evaluated through SDS-PAGE gel electrophoresis.

Protein alkylation, trypsin enzymatic hydrolysis, and TMT tagging

The proteins were alkylated in accordance with Randall's protocol (13), and the filter-aided proteome preparation method was used for protease hydrolysis (14). The trypsin enzyme was added on the basis of the ratio of protein: enzyme = 40:1. The mixture was placed at 37 ℃ overnight. Then, the peptides were desalted and lyophilized. A total of 100 μg protein was taken from each sample for TMT labeling by using the 10-plex TMT reagent (Thermo fisher, Art.No.90111) according to the manufacturer's instructions. Three biological replicates were sampled for each group. The labeling steps were as follows: First, the TMT reagent was allowed to recover to room temperature. Then, acetonitrile was added to the sample, and the sample was centrifuged at low speed with a vortex. Second, the sample was mixed with TMT reagent, incubated at room temperature for 2 h, and then mixed with hydroxylamine. The mixture was reacted at room temperature for 15 min. Finally, the same amount of labeled substances was mixed in a tube and drained with a vacuum concentrator.

HPLC fractionation and LC–MS/MS analysis

Polypeptide samples were redissolved with UPLC loading buffer, and a reverse phase C18 column was used to separate the high pH liquid phase. A total of 20 fractions were collected and merged into 10 fractions in accordance with peak type and time. After vacuum centrifugation and concentration, the mass spectrometry sample was dissolved with the loading buffer solution for mass spectrometry. The mass spectrometry conditions were as follows: The data acquisition software was Thermo Xcalibur 4.0 (Thermo, USA). The chromatographic instrument was Easy NLC 1200 (Thermo, USA), and the mass spectrometer was Q_Exactive HF-X (Thermo, USA). The chromatographic separation time was 120 min, the flow rate was 300 nL/min, the scanning range of MS was 350–1300 m/z, and the acquisition mode was data-dependent acquisition (DDA).

Bioinformatic analysis

Proteome Discoverer^{TM} software 2.4 was used to search the Eubrachyura Uniprot database and our assembled reference transcriptome (15) to identify and quantify proteins. The MS/MS search criteria were as follows: Mass tolerance of 20 ppm for MS and 0.02 Da for MS/MS Tolorance, trypsin as the enzyme with two missed cleavage allowed, carbamido methylation of cysteine and the TMT of N- terminus and lysine side chains of peptides as fixed modification, and methionine oxidation as dynamic modifications, respectively. False discovery rate (FDR) of peptide identification was set as $P \leq 0.01$. A minimum of one unique peptide identification was used to support protein identification. Proteins with fold change (FC) < 0.667, or FC >1.5, and $P < 0.05$ for the FDR were considered as differentially expressed proteins. Pairwise comparison was conducted between every two groups. Differentially expressed proteins were subjected to Gene Ontology (GO) and Kyoto Encyclopedia of Genes and Genomes (KEGG) enrichment analysis by using the software implemented in Majorbio I-Sanger Cloud Platform with corrected $P < 0.05$.

Results

Overview of total identified proteins

After submitting the original data file of the mass spectrometer off machine to the Proteome Discoverer server, 358,336 secondary spectra were obtained, 67,127 spectra were matched, 24,744 peptide fragments, and 9,959 proteins were identified (Fig. 1A). Most identified protein contained less than 23 peptides (Fig. 1B). The protein molecular weight was mainly ranged from 1-80 kDa (Fig. 1C). The error distribution between the real value and the theoretical value of the relative molecular weight of all matched peptides was shown in figure 1D. After the functional annotation of the identified proteins, 4,277 annotated proteins were obtained. Among these proteins, 2,532 proteins were annotated by GO database, accounting for 59.2% of the total annotated proteins, and 3,041 were annotated by KEGG database accounting for 71.1% of the total annotated proteins (Table 1).

Bioinformatic analysis of differentially expressed proteins

The hepatopancreatic proteomics of the three groups were compared and analyzed. A total of 323 differentially expressed proteins were identified with the statistical thresholds of $P < 0.05$, FC > 1.5, or FC < 0.67. Compared with those in group B, 78 proteins were upregulated, and 32 proteins were downregulated in group A (Fig. 2, Table S1). Compared with those in group C, nine were upregulated and 14 were downregulated in group A (Fig. 2, Table S2). There were 190 differentially expressed proteins between groups B and C, and 139 were upregulated and 51 were downregulated in group C (Table S3).

GO enrichment analysis indicated that the differentially expressed proteins between groups A and B were mainly enriched in hydrolase activity, deacetylation, lipoprotein metabolism, and galactosidase activity (Fig. 3, Table S4). The differentially expressed proteins between groups A and C were mainly enriched in oxidative stress reaction and amino acid metabolism (Fig. 3, Table S5). The differentially expressed proteins between groups B and C were mainly enriched in protein modification and hydrolase activity (Fig. S1, Table S6). KEGG database was used to analyze the enrichment of differentially expressed proteins in metabolic pathways. The results showed that the differentially expressed proteins between groups A and B were mainly enriched in lysosomes, sphingolipid metabolism, and polysaccharide degradation pathways (Fig. 4, Table S7), whereas the differentially expressed proteins between groups A and C were mainly enriched in metabolic pathways related to infection (Fig. 4, Table S8). The differentially expressed proteins between groups B and C were mainly enriched in lysosome, homologous and N-glycan biosynthesis pathway (Table S9, Fig. S2)

Differentially expression proteins between groups

The proteomes of groups A and B were compared and analyzed. The proteins that were upregulated in group A included cryptocyanin, cuticle protein, solute carrier family 35 member F6, programmed cell death protein, fibroblast growth factor receptor 3. The downregulated proteins in group A were ataxin-2, metalloreductase, pancreatic lipase-related protein 2, and arylsulfatase A (Table 2). The proteomes of groups A and C were also compared and analyzed. Mannosyl oligosaccharide glucosidase, glutathione

peroxidase 2, calreticulin were among the proteins that were upregulated in group A relative to group C. The proteins that were downregulated in group A were myosin and Rho-associated protein kinase 2 (Table 3).

Discussion

Aquatic plants and freshwater snails are important natural food sources for *E. sinensis* and have a direct effect on the growth and development of *E. sinensis* (16, 17). This study was conducted to investigate the differences in the hepatopancreatic proteomes of *E. sinensis* under three different feeding methods: aquatic plants combined with freshwater snails (group A), aquatic plants only (group B), and freshwater snails only (group C). The results showed differences in the protein expression profiles in the hepatopancreas of *E. sinensis* among the three groups. Meanwhile, our previous study has indicated that the weight gain rate and shell length gain rate was highest in group A than in the other two groups ($P < 0.05$), and lower condition factor and hepatopancreas index were identified in group B than groups A and C (18). Together, this study indicated different feed types may alter the proteome of hepatopancreas and affect the growth and development of *E. sinensis*.

Among the differentially expressed proteins between groups A and B, the proteins with high expression levels and the most significant differences in group A were cryptocyanin and cuticle protein CBM. Cryptocyanin is an important member of the hemocyanin gene family and a crustacean molting protein that is closely related to molting and plays an important role in new exoskeleton formation after molting (19, 20). Cuticle proteins are essential component of the exoskeleton of crustacean species. During molting period, the old epidermis falls off, and a new epidermis will be formed in pre-molt stage. Cuticle protein plays a vital role in the formation of the epidermis during molting (21). Cryptocyanin and cuticle protein expression levels were significantly higher in *E. sinensis* fed with aquatic plants and freshwater snails than in those fed with aquatic plants only, indicating that the molting frequency of *E. sinensis* fed with aquatic plants and freshwater snails might be accelerated; this result was also consistent with our previous research results showing that the molting rate of *E. sinensis* fed with aquatic plants and freshwater snails is significantly faster than that of *E. sinensis* fed with aquatic plants only (18). The results of this study showed that the addition of snails to the daily diets of *E. sinensis* could affect the expression of molting-related proteins and further affect the moltingprocess. A large number of proteins related to cell proliferation and growth were highly expressed in *E. sinensis* fed with aquatic plants and freshwater snails; these proteins included solute carrier family 35 member F6 (22), programmed cell death protein 2(23, 24) , and UCN-45 protein homolog A (25) (Table 2). The high expression of these proteins leads to the increase in cell number and volume and further affects the growth and development of *E. sinensis*. Therefore, *E. sinensis* fed with aquatic plants and freshwater snails grew significantly faster than *E. sinensis*fed with aquatic plants only as our previous study presented (18).

Among the proteins that were differentially expressed between groups A and C, those that were highly expressed in group A were mannosyl oligosaccharide glucosidase, glutathione peroxidase-2, and calreticulin. Mannosyl oligosaccharide glucosidase is involved in the metabolism of mannan oligosaccharides and can improve the immunity in *Litopenaeus vannamei* (26, 27). Glutathione peroxidase plays an important role in immune defense against pathogen infection in invertebrates. Research on

Haliotis discus, *Chlamys farreri*, *L. vannamei*, and *Fenneropenaeus chinensis* has shown that glutathione peroxidase is involved in the immune regulation process (28-31). Calreticulin is a highly conserved calcium binding protein, which is an immune-related protein in vertebrates and invertebrates. Studies on *Patinopecten yessoensis*, *Sebastes schlegeli*, and *Tilapia niloticus* all showed that calreticulin is involved in immune regulation (32-34). The levels of these proteins were higher in *E. sinensis* fed with aquatic plants and freshwater snails than in *E. sinensis* fed with only freshwater snails, suggesting that aquatic plants might affect the immunity of *E. sinensis*. Moreover, these results were consistent with previous results that submerged plants in the diet can enhance the immunity of *E. sinensis* (35, 36).

Conclusions

Diets containing aquatic plants may enhance the immunity of *E. sinensis*, while those containing freshwater snails may promote the growth and molting of *E. sinensis*. A mixed diet containing both aquatic plants and freshwater snails maybe the best choice for *E. sinensis*. The results of this work provide a theoretical basis for the practice of “providing aquatic plants and freshwater snails” in the ecological culture of *E. sinensis.*

Declarations

Ethics approval and consent to participate

The study was approved by the Institutional Animal Care and Use Committee of Shanghai Ocean University (Shanghai, China). Sampling procedures complied with the guidelines of the Institutional Animal Care and Use Committee on the care and use of animals for scientific purposes. **Acknowledgements**

Not applicable.

Consent for publication

Not applicable.

Availability of data and materials

All data used to support the findings of this study are available upon request to the corresponding author. The dataset generated in this study has been deposited in the ProteomeXchange Cosortium (http://proteomecentral.proteomeexchange.org) with the dataset identifier px-submisssionPXD023215.

Competing interests

All authors declare that they have no competing interests.

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Authors' contributions

JW and CHW conceived the original idea of the study; XWC, XH, ZHL and DYY performed experiments; JW, and XWC analyzed data; XWC and XH interpreted results; XWC and XH prepared figured and drafted manuscript; JW and CHW edit and revised manuscript. XWC and XH approved final version of manuscript. XWC and XH contributed equally to this work.

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Tables

Table1. The information of identified protein with annotation based on different database.

Databases	Number of proteins	Percentage
SubCell-Location	4277	1
COG	1481	0.3463
KEGG	3041	0.711
GO	2532	0.592
Pfam	3844	0.8988
Total	4277	1

Percentage: Ratio of number of annotated proteins to the total number of proteins.

Table 2. Important differentially expressed proteins between the hepatopancreas of *E. sinensis* in group A fed with *E. canadensis* and *S. quadrata* and in group B fed with *E. canadensis* only ($P < 0.05$, FC < 0.67, or FC > 1.5,).

Accession/ Uniprot	Protein	P value	Coverage [%]	No. of peptides	Fold change A/B	Related Function
A0A223G1B9	Putative hemocyanin	0.002733	16.00587	3	3.83	Molt and growth
O96992	Putative hemocyanin	0.02816	23.583461	1	6.67	Molt and growth
A1YLE8	Cuticle protein CBM	0.02132	16.51376	1	2.26	Cuticle
Q8N357	Solute carrier family 35 member F6	0.04072	1.745636	1	1.70	Cell proliferation
P47816	Programmed cell death protein 2	0.002475	8.241758	3	1.77	Cell proliferation
Q99KD5	Protein unc-45 homolog A	0.04481	1.740812	1	1.73	Cell proliferation
O00571	ATP-dependent RNA helicase DDX3X	0.01342	16.19813	11	1.51	Cell growth
Q14678	KN motif and ankyrin repeat domain-containing protein 1	0.001738	2.578797	1	1.50	Cell proliferation
Q6NX65	Programmed cell death protein 10	0.04888	4.587156	1	1.76	Cell proliferation
O70305	Ataxin-2	0.02852	0.64695	1	0.46	Negative regulation of growth
Q687X5	Metalloreductase	0.00888	1.520913	1	0.64	Negative regulation of cell proliferation
P12617	Malonyl-CoA decarboxylase	0.0351	1.666667	1	1.60	Lipid synthesis
Q99JB2	Stomatin-like protein 2	0.009692	26.36986	3	1.62	Lipid localization
A0A0P4VPE9	FABP domain-containing protein	0.03487	11.19403	1	1.58	Lipid binding
Q9NUQ2	1-acyl-sn-glycerol-3-phosphate acyltransferase epsilon	0.04965	1.715686	1	1.50	Phospholipid synthesis
P57093	Phytanoyl-CoA	0.01746	17.64706	1	1.65	Lipid

	dioxygenase					metabolism
Q91XV4	L-xylulose reductase	0.006572	70.78189	13	0.60	Carbohydrate metabolism
Q95327	β-mannosidase	0.04392	5.925926	1	0.47	Carbohydrate metabolism
P54318	Pancreatic lipase-related protein 2	0.03813	14.72393	2	0.32	Nutrition response
P50428	Arylsulfatase A	0.0166	10.91703	2	0.58	Nutrition response
P10768	S-formylglutathione hydrolase	0.005636	42.76923	12	1.78	Thiolester hydrolase activity
K7IXU0	Palmitoyl-protein hydrolase 1	0.01932	20.80537	2	0.66	Lipoprotein metabolic process
Q9TRY9	Beta-galactosidase	0.02479	43.94905	4	0.64	Beta-galactosidase activity
A0A0P4WEX6	Palmitoyl-protein hydrolase 1	0.04374	9.666667	1	0.48	Protein deacylation

Table 3. Important differentially expressed proteins between the hepatopancreas of *E. sinensis* in group A fed with *E. canadensis* and *S. quadrata* and group C fed with *S. quadrata* only ($P < 0.05$, FC < 0.67, or FC > 1.5,).

Accession/ Uniprot	Protein	P value	Coverage (%)	No. of peptides	Fold change A/C	Related Function
Q80UM7	Mannosyl-oligosaccharide glucosidase	0.02686	17.01031	1	1.52	Immune
Q4AEI0	Glutathione peroxidase 2	0.0428	35.50725	3	1.62	Immune/Response to oxidative stress
A0A193DUV8	Calreticulin	0.01644	67.9803	2	4.50	Immune
P05661	Myosin heavy chain	0.04912	3.655352	1	0.61	Muscle contraction
M3TYT0	Rho-associated protein kinase 2	0.01186	1.763409	2	0.64	Muscle contraction
O43396	Thioredoxin-like protein 1	0.002447	29.67033	5	0.55	Response to oxidative stress
Q6NWZ9	Cysteine dioxygenase type 1	0.03732	16.74641	4	0.56	L-cysteine metabolic process

Figures

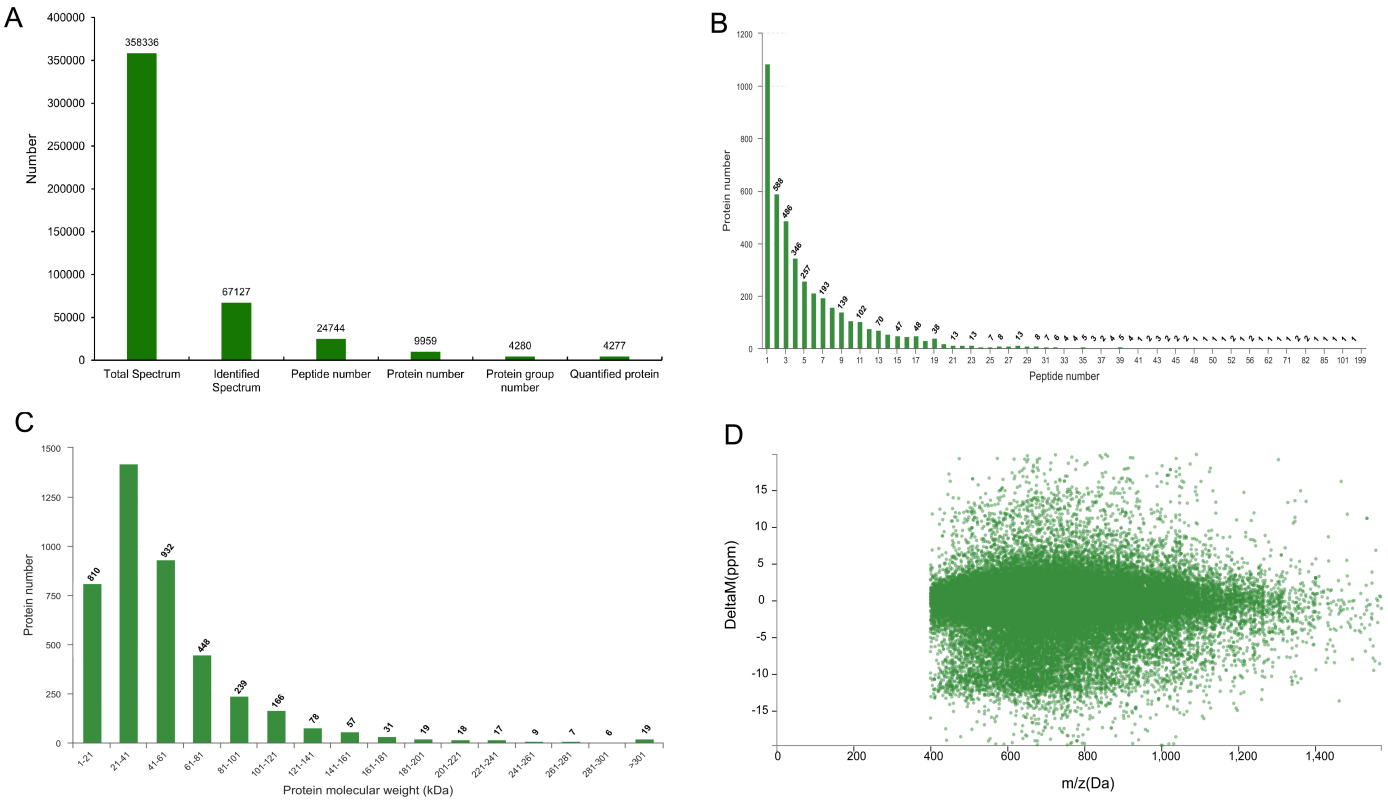

Figure 1

Statistical data of reliable peptides in hepatopancreas samples of E. sinensis. A. The distribution of peptide matching error. B. The distribution of peptide number. C. The distribution of protein molecular weight. D. The number of proteins.

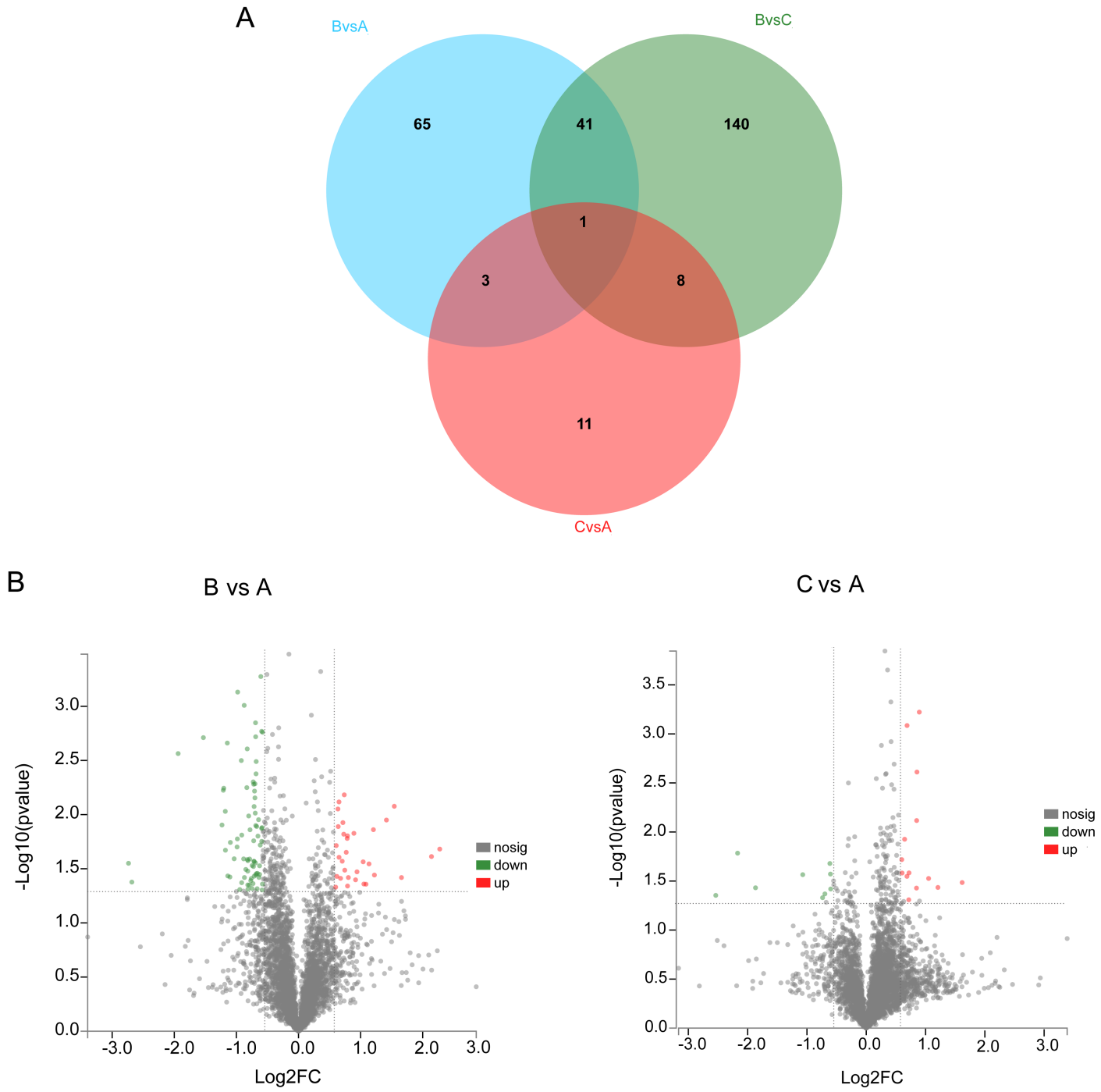

Figure 2

The display of differentially expressed genes. A. Venn diagram of differentially expressed genes between groups. B. Volcano map of differentially expressed proteins between groups. P < 0.05, Fold change >1.5 or <0.67. The green means down-regulated proteins and red means up-regulated proteins.

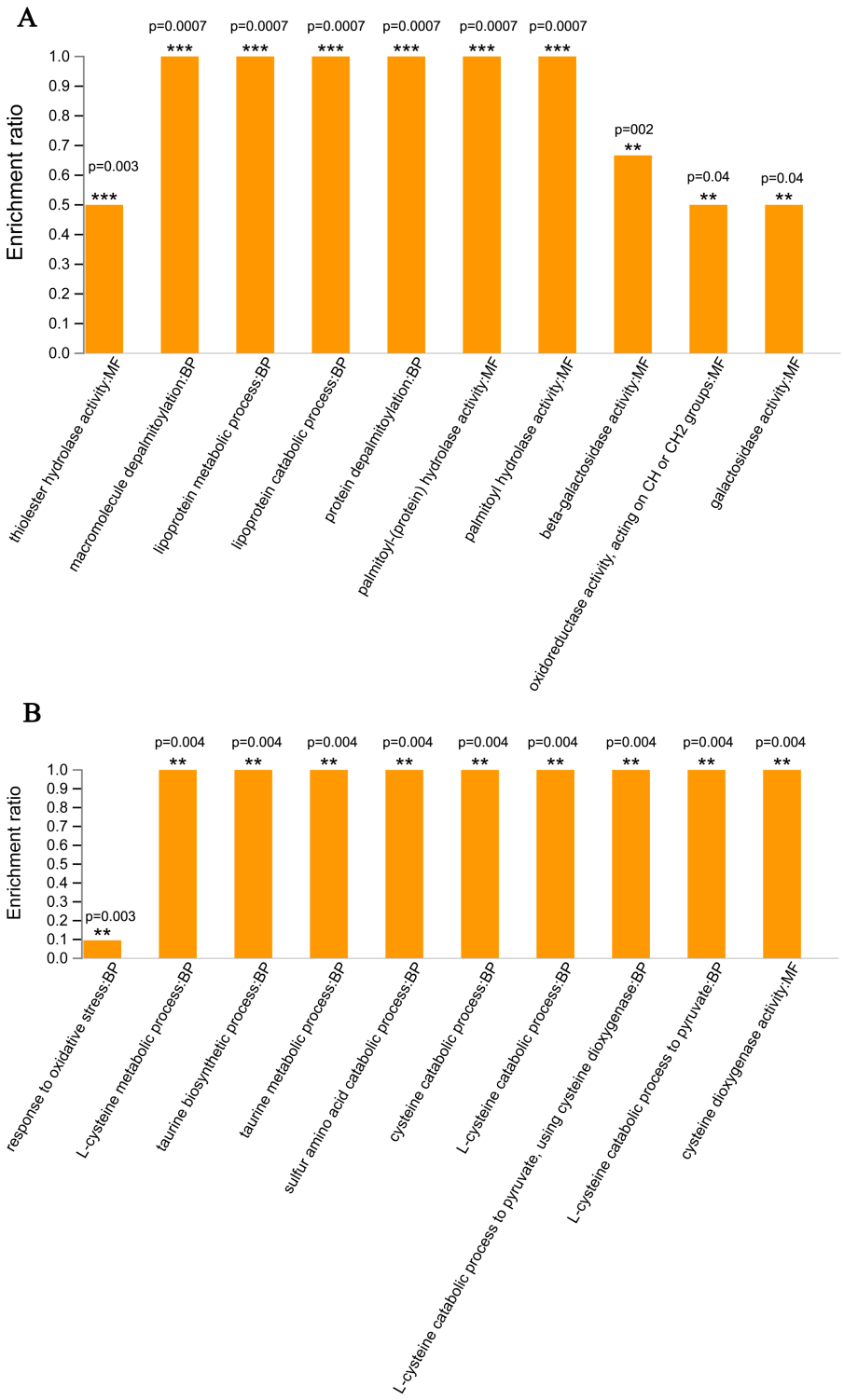

Figure 3

Gene ontology (GO) annotation of differential expressed proteins between groups A and B (A) and between groups A and C (B). Note: *** P < 0.001, ** P < 0.01, * P < 0.05.

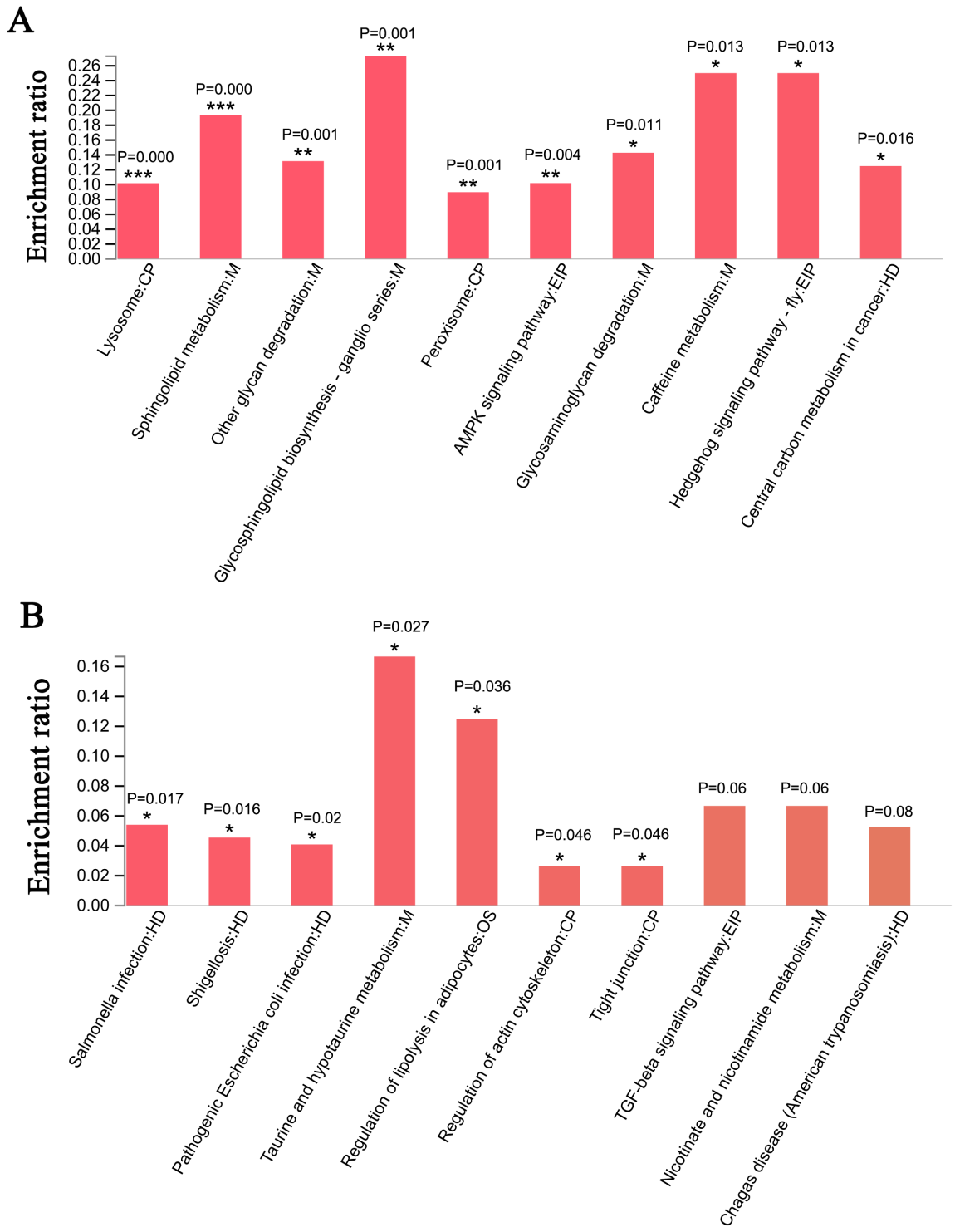

Figure 4

KEGG pathway enrichment analysis of differential expressed proteins between groups A and B (A) and between groups A and C (B). Note: *** P< 0.001, ** P < 0.01, * P < 0.05.

Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.

- TableS1.xls
- TableS2.xls
- TableS3.xls
- TableS4.xls
- TableS5.xls
- TableS6.xls
- TableS7.xls
- TableS8.xls
- FigureS1.tif
- FigureS2.tif