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. quadrata* and *E. canadensis*, group B was fed with *E. canadensis* only, and group C was fed with *S. quadrata* 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 °C ± 2 °C, 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 replicates were collected for each group. Then, the tissues were quickly frozen in liquid nitrogen and stored in a −80 °C 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 °C and 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 °C 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™ 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 Tolerance, 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)

**Differently 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 molting process. 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 Consortium ([http://proteomecentral.proteomeexchange.org](http://proteomecentral.proteomeexchange.org)) with the dataset identifier px-submissionPXD023215.

**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 contributed equally to this work.

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**Tables**

Table 1. 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 | 0.001738 | 2.578797   | 1               | 1.50            | Cell proliferation        |
| Q6NX65            | Programmed cell death protein 10       | 0.04888   | 4.587156   | 1               | 1.76            | Cell proliferation        |
| 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 dioxygenase              | 0.01746   | 17.64706   | 1               | 1.65            | Lipid metabolism          |
| Q91XV4            | L-xylulose reductase                   | 0.006572  | 70.78189   | 13              | 0.60            | Carbohydrate metabolism   |
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              |