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Effects of dietary chromium on growth, amino acid content and proteomic changes in Sea Cucumber *Apostichopus japonicus*

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To assess the effect of dietary chromium, the growth rate, amino acid content and proteomic changes in the sea cucumber *Apostichopus japonicus* were compared, when fed with the diets amended with chromium-treated (58.27 mg Cr/kg dry weight) *Macrocystis pyrifera* and a Cr-free control diet (5.83 mg Cr/kg dry weight). After 10 days, the dietary chromium exposure decreased its growth rate and the amino acid content also changed. The proteomic changes were analyzed in *A. japonicus* after it was fed for 10 days with Cr-added and Cr-free control diet. The total of 1587 proteins were identified, of which 28 proteins were identified as differentially regulated proteins in sea cucumber to Cr stress. Among them, 10 proteins were identified. In summary, this work reported toxic effects in sea cucumber *A. japonicus* after dietary exposure to Cr.

[Keywords: Apostichopus japonicus; Chromium; Macrocystis pyrifera; Proteomic changes]

Introduction

Excess of heavy metals due to their elevated release mainly by industry becomes an important ecological problem. The heavy metal contamination enters the aquatic environment from farms, urban and industrial production sites, and cause long-term eco-toxicological effects as they could also exert deleterious effects on animal and human health[1]. Heavy metals are toxic, persistent, and non-biodegradable which would be inevitably moved through food chains[2]. The Cr compounds cause environmental pollution as a result of a large number of industrial operations, including mining, pigment manufacturing, petroleum refining, leather tanning, wood preserving, textile manufacturing, pulp processing and fungicide development[3]. It exists in nature in both trivalent and hexavalent forms, of which the latter is more toxic. Of them, Cr (^{o}C) is an essential micronutrient for animals at low levels, whereas Cr (^{o}C) is seriously toxic for both animals and plants[4]. Moreover, Cr (^{o}C) is highly hazardous to human health and can cause dermatosis, lung cancer, pulmonary and upper respiratory tract disorders, nephrotoxicity, and digestive and teratogenic effects. Therefore, bioaccumulation of toxic heavy metals in food chain has become a global health concern. Cr contamination is posing a serious threat to the environment, emerging as a major health hazard to the biota. Despite the widespread occurrence of Cr toxicity, its molecular mechanism is poorly documented in animals.

Proteomics is a novel methodology and provide unique insights into biological systems that cannot be afforded from genomic or transcriptomic approaches. Proteomics-based biomarker discovery has predominantly focused on identifying different proteins that are expressed in a given organ, tissue or cell line under abiotic stresses including heavy metal stress[5]. TMT reagents are often used for discovery studies to reveal proteins being differentially expressed between individuals and groups differing in physiological status[6,7]. Sea cucumber *A. japonicus* is an edible marine species in China. The comparative proteomic analysis of the global protein expression changes in *A. japonicus* under abiotic stresses. Forty different proteins were identified in *A. japonicus* after the pathogen challenge and 27 protein spots with significant differences in abundance were identified under high temperature stress[8,9]. While, up to now there is no comparative proteomic report on the changes in protein pattern of *A. japonicus* under Cr stress.

Sea cucumber *A. japonicus* is a dominant mariculture species in northern China due to its relatively high economic value. Now, more and more farmers fed the sea cucumbers with macroalgae and sea mud to improve their yield. Heavy metal contamination of diet can change growth performance and antioxidant enzyme activities of sea cucumber[10].

Chromium can be accumulated in the tissues of aquatic plant and biomagnify along the food chain, which results into myriad ecological damages and health risks to ecosystems and humans when concentrations reach certain toxicity thresholds. The risks to humans are most notable when contaminated sea cucumbers are consumed beyond the allowed daily intake levels. In the present study, the effects of dietary Cr on growth rate, amino acid content and proteomic changes in sea cucumber *A. japonicus* were studied. The purpose was to obtain insights into the molecular mechanism of Cr toxicity to *A. japonicus*.

Materials and Methods

Diet preparation

M. pyrifera used in this experiment was cultured at Weihai of China. It was washed with seawater for three times and treated with 0 or 20mg.L^{-1} $K_2Cr_2O_7$ for 24 h. The alga was washed with distilled water and dried at 60 ^oC for 48 h. After that, dried alga was ground and sieved using a 0.15 mm mesh. The sand of sea was collected from Huiquanwan, Qingdao. The alga and sand of the sea were mixed in the ration of 7:3. Chromium content was 5.83 and 58.27 mg/kg dry weight in the basal diets and treatment, respectively.

Feeding trial sea cucumber juveniles used in this experiment were obtained from Qingdao Agriculture University. Sea cucumber juveniles of about 7.5±0.2 g were fed with control diet for 18 days before the start of the experiment to acclimatize the sea cucumbers to the diets and to the experimental conditions. Sea cucumbers (7.5±0.2 g) were allocated into 40-L tanks at a density of 10 sea cucumbers per tank with 24 h air supply to maintain dissolved oxygen near saturation. The experiment was performed in a static aquarium system. Water temperature ranged from 10.0 to 12.0 ^oC. The pH was 7.9–8.1 and the salinity was 3.1–3.2%. Sea cucumbers were fed at an amount of 3% of body weight once a day at 16:00. To ensure water quality, 50% of the water was replaced every day and residual diet and feces were removed using a siphon before feeding. The experiment lasted for 10 days. The sea cucumbers were dissected on ice to obtain the body wall and intestines. The body wall and intestines were collected for Cr concentrations. The body wall was collected and dried using vacuum freeze-drying technology for amino acid analysis.

Growth performance

The sea cucumbers were starved for 24 h after fed for 10 days, and then every sea cucumber was weighed to determine final body weight (FBW) and specific growth rate (SGR). Growth parameters were calculated as follows:

$$SGR\ (\%.\ d^{-1}) = 100 \times (\ln W_2 - \ln W_1)/T$$

Where, W_2 and W_1 were the final and initial dry body weights of the sea cucumber (g), respectively. T was the duration of the experiment (d).

Chromium, calcium and magnesium content

The body wall and digestive extracts were separated and rinsed in distilled water for three times. Then the samples were dried at 60 ^oC for 48 h and incinerated at 500 ^oC for 5 h in muffle. The obtained ash was treated with concentrated hydrochloric acid and the content of Cr, calcium (Ca) and magnesium (Mg) were analyzed using atomic absorption spectrophotometer (AAS). The concentrations in the tissues were expressed as mg/ Kg.

The amino acid analysis

For free amino acid, the extraction solvent was 0.02 mol l^{-1} HCl. Extraction was performed three times and each extract was sonicated for 15 min. The equal volume of 3% 5-sulfosalicylic acid was then added into the homogenate for deproteinization, which was maintained at 4 ^oC for 15 h. After that, the homogenate was centrifugated at 15,000 g for 10 min at 4 ^oC, and filtered through a 0.2 μm filter. The free amino acid contents of the filtrate were determined using an amino acid analyzer. The amino acid standard solution was used for identification and quantification of free amino acids. The content of amino acid was expressed as mg/g.

Statistics analysis

Results were presented as means± SD. One-way analysis of variance was performed to determine statistical differences between two groups. When overall differences were significant ($\rho < 0.05$), Duncan's multiple range test was used to compare significant differences between two groups. Statistical analyses were performed using SPSS 16.0 for Windows.

The sample preparation and TMT labeling

The body wall of sea cucumber was separated and pulverized in liquid nitrogen and then 1 ml lysis buffer containing 7M urea, 4% SDS and 1x protease inhibitor cocktail (Roche Ltd. Basel, Switzerland) was added. The samples were subjected to ultrasonication and centrifuged at 20,000 g for 10 min at 4 ^oC. The supernatant was collected for determining the protein concentration. 100 μg proteins per sample were

measured by Bradford assays and 1M DTT was incubated at 55 °C for 1 hour, then alkylated with 600 mM iodoacetamide for 30 min in dark. The proteins of each sample were precipitated with ice-cold acetone and re-dissolved in 100 μL dissolution buffer (100 mM TEAB, 1% SDS).

The digested samples with sequence-grade modified trypsin (Promega) were labeled using chemicals from the TMT reagent kit. The sea cucumber fed with Cr-treated *M. pyrifera* were labeled with the tags TMT-126 and TMT-127, while the sea cucumber fed with untreated *M. pyrifera* were labeled with the tags TMT-128 and TMT-129. After tagging, the four samples were pooled and desalted using C18 SPE column (Sep-Pak C18, Waters, Milford, MA) and dried under vacuum[11].

High pH reverse phase separation and low pH nano-HPLC-MS/MS analysis

The peptide mixture was re-dissovled in the buffer A (buffer A: 10 mM ammonium formate in water, pH10.0), and fractionated with high pH separation using Aquity UPLC system (Waters Corporation, Milford, MA) connected to a reverse phase column (BEH C18 column, 2.1mm x 150 mm, 1.7 μm, 130 Å, Waters Corporation, Milford, MA). The linear gradient from 0% B to 45% B in 35 min (B: 10 mM ammonium formate in 90% ACN, pH 10.0) was used in high pH separation with a flow rate of 250 μL/min at 45 °C.

Each of the dried fractions was dissolved in 32 μl A and B, respectively to get A/B mixture (A: ddH_2O with 0.1% formic acid, B: ACN with 0.1% formic acid). After that, the samples were separated by nanoLC and analyzed by online electrospray tandem mass spectrometry. The EASY-nLC 1000 system (Thermo Fisher Scientific, Waltham, MA) connected to an Orbitrap fusion mass spectrometer (Thermo Fisher Scientific, San Jose, CA) equipped with an online nano-electrospray ion source were used in this experiment. 6 μl peptide sample was loaded onto the trap column (Thermo Scientific Acclaim PepMap C18, 100 μm x 2 cm) with a flow of 10 μl/min for 3 min and then separated on the analytical column (Acclaim PepMap C18, 75μm x 25cm) with a linear gradient started from 2% D to 40% D in 105 min. The column was re-equilibrated at initial conditions for 15 min with a flow rate of 300 μL/min. The electrospray voltage of 2.0 kV versus the inlet of the mass spectrometer was used. The Orbitrap fusion mass spectrometer was conducted in the data-dependent mode to switch automatically between MS and MS/MS acquisition.

Database searching

The tandem mass spectra were analyzed by proteome discoverer software (Thermo Fisher Scientific, version 1.4.0.288). Charge state deconvolution and deisotoping were not performed. All MS/MS samples were analyzed using Mascot (Matrix Science, London, UK; version 2.3), which was set up to search the self database assuming the digestion enzyme trypsin. Mascot was searched with a fragment ion mass tolerance of 0.050 Da and a parent ion tolerance of 10.0 PPM. Carbamidomethyl of cysteine and TMT 6 plex of lysine and the n-terminus were specified in Mascot as fixed modifications. Oxidation of methionine was specified in Mascot as a variable modification.

Quantitative data analysis

The percolator algorithm was used to control peptide level false discovery rates (FDR) lower than 1%. Only unique peptides were used for protein quantification, and the method of normalization on protein median was used to correct experimental bias, the minimum number of proteins that must be observed to allow was set to 1000. The statistically significant enrichment was determined using Fisher's exact test. Significant enrichments were selected when the Benjamini-Hochberg's p value was adjusted as $\rho<0.05$. Gene Cluster 3.0 software was operated to construct hierarchical clustering for the differentially changed proteins.

Results

Cr, Ca and Mg accumulation

The Cr, Ca and Mg accumulation in intestine and body wall of sea cucumber is shown in Figure 1. The content of Cr was increased in sea cucumber fed with Cr-adding *M. pyrifera*. In the aquatic environment, the absorption of Cr in sea cucumber occurred via the intestines from contaminated food, and the intestines for diet exposure were major metal uptake. Therefore, the accumulation of Cr was higher in intestines than the body wall (Table. 1).

Growth

No mortality was observed for the exposure periods. The influence of dietary Cr supplementation on growth performance in the exposure experiment is shown in Table 2. The relatively growth rates of sea cucumber were 0.44% and 0. 27% under Cr-added diet and Cr-free control diet, respectively.

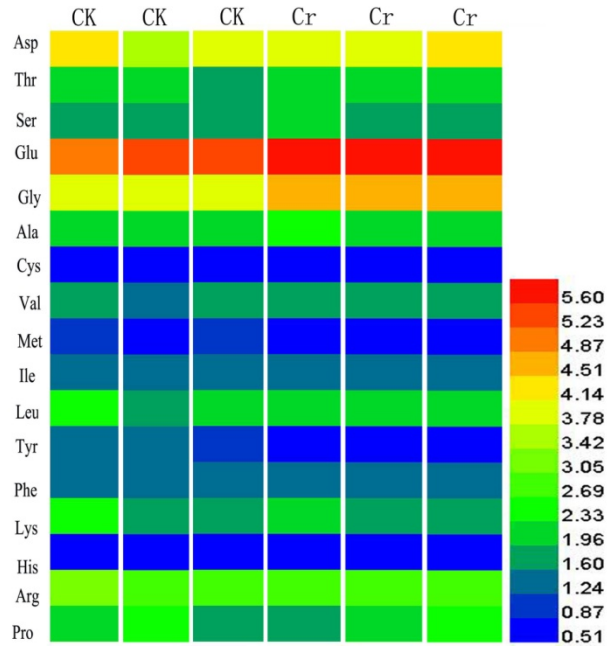

Fig. 1 — Hierarchical clustering of changes in abundance of amino acid in sea cucumber under Cr-added diet and Cr-free control diet. Red color and blue color represented higher and lower content of amino acid, respectively. Values were expressed as mean±SD (n =3). The unit was mg g^{-1}. CK and Cr represented sea cucumber under Cr-free control diet and Cr-added diet.

This experiment demonstrated that 10-day high concentration Cr-supplemented diet decreased the SGR of sea cucumbers as shown in Table 2.

The amino acid contents

The total free amino acid composition in each sea cucumber was ascertained by the amino acid analyzer. The hierarchical clustering patterns (heat map) of amino acid content from sea cucumber fed Cr- adding and Cr-free *M. pyrifera* is illustrated in Figure 1. The content of glutamate (Glu) and glycine (Gly) were significantly increased. The content of Glu and Gly enhanced 11.93% and 14.12%, respectively. Whereas the content of tyrosine (Tyr) and methionine (Met) clearly decreased. The content of Tyr and Met reduced 21.11% and 42.97%, respectively.

The differently expressed proteins

The differently expressed proteins were identified by TMT in sea cucumber *A. japonicus*. To investigate this issue, differentially expressed proteins in the two groups (126,127 group and 128,129 group) were identified as significantly changed compared to the sea cucumber fed with Cr-adding and Cr-free macroalgae. A total of 28 proteins were identified that were differentially expressed in under Cr-added diet and hierarchical clustering patterns (heat map) were used to illustrate the changing abundances of differentially expressed proteins from each group (Fig. 2). A total of 28 proteins were identified that were differentially expressed in Cr-added sea cucumber. The identification of some Cr-responsive proteins might provide new insights to the heavy metal toxicology.

Table 1 — Total Cr, Ca and Mg accumulated in sea cucumber under Cr-added diet and Cr-free control diet.

	Cr (μg/L)	Ca (mg/L)	Mg (mg/L)
CK (body wall)	1.90±0.82	13.13±1.20	11.03±0.93
Cr-treatment (body wall)	4.89±0.4*	6.89±0.53*	10.98±0.59
CK (intestine)	2.68±0.41	15.75±1.21	11.98±0.83
Cr-treatment (intestine)	8.07±0.77*	4.87±0.900*	5.35±0.43*

Values were expressed as mean±SD (n =3). The means were compared using the student *t* test (*P<0.05)

Table 2 — Effects of dietary Cr on the growth of sea cucumber under Cr-added diet and Cr-free control diet

	IBM(g)	FBM(g)	SGR (%/day)
CK	7.51±0.07	8.31±0.20	0.44±0.06
Cr-treatment	7.54±0.05	8.02±0.10*	0.27±0.03*

Values were expressed as mean±SD. (n =3). The means were compared using the student t test (*P<0.05). IBW: initial body weight; FBW: final body weight; SGR (specific growth rate) = 100× (ln final mean weight−ln initial mean weight)/no. of days.

Discussion

The results of the present study demonstrated that the dietary Cr exposure to sea cucumber induces a significant chromium accumulation in gut and body wall. The toxic effects of the toxicant exposure on the aquatic animals had been varying with the animals and physico-chemical properties of water, as well as the differences in the kind and exposure time[12]. The accumulation in aquatic animals had different mechanisms via the direct uptake from water by gills and ingestion from food by intestine[13]. It was well known that the heavy metal uptake via food chain in aquatic animals might depend on metal concentrations of food and metabolism[14]. Similar results were concluded in *Oreochromis niloticus* that the accumulation of heavy metals (Al, As, Cd, Cr, Cu, Fe, Mn, Ni, Pb, and Zn) was always higher in the liver than in the muscle[15]. The Ca and Mg were major elements in regulating ion to maintain stable cellular and enzymatic functions, and the values of Ca and Mg could be altered by the toxicity of metallic exposure[16,17]. The content of Ca and Mg was decreased with the increase of Cr in sea cucumber.

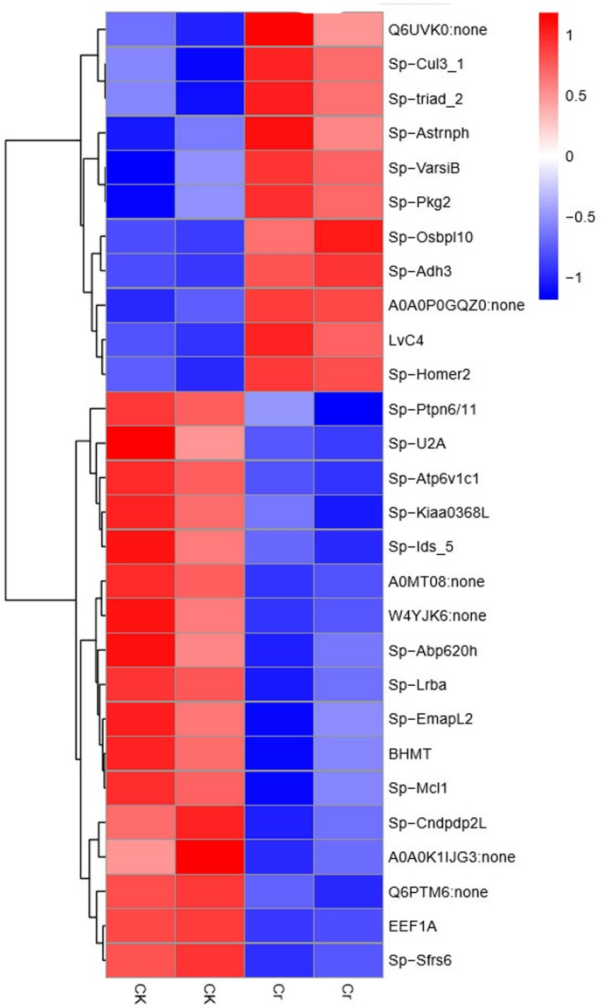

Fig. 2 — Differentially expressed proteins identified by the TMT experiment in body wall of sea cucumber (*Apostichopus japonicus*) under Cr-added diet and Cr-free control diet. Red color and blue color represented higher and lower content of protein, respectively. CK and Cr represented sea cucumber under Cr-free control diet and Cr-added diet.

The inhibition of Mg^{2+} and Ca^{2+} uptake under Cr assimilation was affected by blocking the binding sites of transport proteins.

The dietary Cr exposure had adverse influence on the growth rate. Considering the accumulation in the gut and body wall, inhibition of growth, alterations in amino acid content and proteins, the dietary chromium exposure has a bad effect on sea cucumber as a substantial toxicity. Glutathione, consisting of Glu, Gly and cystine (Cys) played an important role in antioxidant defense and redox regulation. The decrease of Glu and Gly might have inhibited the content of glutathione.

The betaine-homocysteine S-methyltransferase (BHMT gene) catalyzed the synthesis of methionine from betaine and homocysteine[18], and methionine adenosyltransferase synthesize S-adenosylmethionine. In this study, methionine adenosyltransferase and betaine-homocysteine S-methyltransferase obviously decreased in sea cucumber after fed with Cr-adding alga, which further resulted in the decrease of met. Pyruvate kinase (A0A0P0GQZ0 gene) was the thiol-containing enzyme that catalyzed the final step of glycolysis and exerted a key role for cellular energy homeostasis. Lead inhibits in vitro pyruvate kinase activity in brain cortex of rats[19]. While, the PK activity decreased in sea cucumber after it was fed with Cr-adding alga. The difference might be due to different heavy metals and organisms. The increase of PK activity promoted pyruvate levels inside the cells. Pyruvate is an intermediate metabolite of glucose metabolism as an effective scavenger of reactive oxygen species[20]. Pyruvate might induce cell death because it could prevent neuronal death promoted by glycolytic inhibition by Zn[21]. Therefore, simultaneous increase of PK activities could strongly increase energy metabolism and antioxidant defenses (pyruvate). The eukaryotic elongation factor 1 complex (EEF1A gene) bond to and delivers aminoacyl-tRNA to the A-site of ribosomes during protein biosynthesis, which decreased in sea cucumber under Cr stress. While oxysterol-binding proteins had functions in cellular lipid metabolism or sterol transport[22]. Therefore, the assimilation of Cr in sea cucumber might result in the change of protein and lipid.

Conclusion

This experiment demonstrated Cr-induced effects on the growth performance, heavy metal contents, amino acid content and proteomic changes in sea cucumbers. The growth rate decreased with the accumulation of Cr in gut and body wall of sea cucumber. The amino acid content was also changed, and 28 proteins were also identified as differentially regulated proteins in sea cucumber to Cr stress.

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