Comparative Biochemistry and Physiology, Part D 19 (2016) 78–89

Contents lists available at ScienceDirect

Comparative Biochemistry and Physiology, Part D

journal homepage: www.elsevier.com/locate/cbpd

# Understanding mechanism of sea cucumber *Apostichopus japonicus* aestivation: Insights from TMT-based proteomic study

Muyan Chen [a,*], Xingke Li [a], Aijun Zhu [a], Kenneth B. Storey [b], Lina Sun [c], Tianxiang Gao [d], Tianming Wang [e]

[a] Fisheries College, Ocean University of China, Qingdao, PR China
[b] Institute of Biochemistry, Carleton University, 1125 Colonel By Drive, Ottawa, ON K1S 5B6, Canada
[c] Institute of Oceanology, Chinese Academy of Sciences, Qindao, PR China
[d] Fishery College, Zhejiang Ocean University, Zhoushan, PR China
[e] Marine Science and Technology College, Zhejiang Ocean University, Zhoushan, PR China

ARTICLE INFO

*Article history:*
Received 15 December 2015
Received in revised form 31 May 2016
Accepted 14 June 2016
Available online 17 June 2016

*Keywords:*
Aestivation
Sea cucumber
Proteome
Tandem mass tag
Post-transcriptional regulation

ABSTRACT

Marine invertebrate aestivation is a unique strategy for summer survival in response to hot marine conditions. The sea cucumber, *Apostichopus japonicus*, is an excellent model marine invertebrate for studies of environmentally-induced aestivation. In the present study, we used a tandem mass tag (TMT)-coupled LC-MS/MS approach to identify and quantify the global proteome expression profile over the aestivation-arousal cycle of *A. japonicus*. A total of 3920 proteins were identified from the intestine of sea cucumber. Among them, 630 proteins showed significant differential expression when comparing three conditions of sea cucumbers: non-aestivating (active), deep-aestivation (at least 15 days of continuous aestivation), and arousal after aestivation (renewed moving and feeding). Sea cucumbers in deep aestivation showed substantial differentially expressed proteins (143 up-regulated and 267 down-regulated proteins compared with non-aestivating controls). These differentially expressed proteins suggested that protein and phospholipid probably are major fuel sources during hypometabolism and a general attenuation of carbohydrate metabolism was observed during deep aestivation. Differentially expressed proteins also provided the first global picture of a shift in protein synthesis, protein folding, DNA binding, apoptosis, cellular transport and signaling, and cytoskeletal proteins during deep aestivation in sea cucumbers. A comparison of arousal from aestivation with deep aestivation, revealed a general reversal of the changes that occurred in aestivation for most proteins. Western blot detection further validated the significant up-regulation of HSP70 and down-regulation of methyltransferase-like protein 7A-like in deep-aestivation. Our results suggest that there is substantial post-transcriptional regulation of proteins during the aestivation-arousal cycle in sea cucumbers.



## 1. Introduction

Global environmental change is a major threat to marine ecosystems. The long-term persistence of species in the face of climate change depends on the ability of populations to keep pace with changing climates or adapt to changes in situ (Burrows et al., 2011). Since most marine ectotherms are thermal range conformers, they are more sensitive to warming than endotherms (Sunday et al., 2012). Aestivation, as an energy-saving strategy and adaptive mechanism of animals, is important for surviving long-term high temperatures or arid conditions and has been reported for multiple terrestrial and freshwater vertebrate and invertebrate model animals (Fuery et al., 1998; Ramos-Vasconcelos and Hermes-Lima, 2003; Ramnanan and Storey, 2006; Malik and Storey, 2009; Salway et al., 2010; Loong et al., 2012; Giraud-Billoud et al., 2013). However, aestivation is understudied in the marine context. The sea cucumber, *Apostichopus japonicus*, is an excellent model organism for studies of environmentally-induced aestivation by a marine invertebrate, and studies of this species are important not only to gain information about the potential effects that climate change could have on this and other marine animals but also because *A. japonicus* is an important food species that is widely grown in aquaculture in Asia, and a major decrease in body weight occurs during aestivation has serious impacts on production efficiency in aquaculture systems.

These sea cucumbers survive extended periods of high water temperatures (lasting up to 100 days) by aestivating during which they cease feeding and locomotion, and undergo metabolic rate depression, organ atrophy, and immune system modification. Although the phenomenon of aestivation in sea cucumbers was first reported over a century ago (Mitsukuri, 1897), research on the mechanisms involved in sea cucumber aestivation has only surged in recent years. Studies have

* Corresponding author.
*E-mail address:* chenmuyan@gmail.com (M. Chen).

http://dx.doi.org/10.1016/j.cbd.2016.06.005
1744-117X/

shown that aestivation in *A. japonicus* is profiled as follows: depressed metabolic rate (lower oxygen consumption and ammonia excretion rate), energetic adjustments (feeding and defecation stop and fuels stored in the body are used to maintain basic physiological activities such as respiration and excretion) (Yang et al., 2006), and degradation of intestinal structure and function (major gastrointestinal atrophy and halting digestive enzyme activity) (Gao et al., 2008). New techniques in molecular biology and bioinformatics have also been applied to understand the molecular mechanisms of sea cucumber aestivation. Our previous studies suggested that microRNAs could play important roles in transcriptional depression and cell differentiation during sea cucumber aestivation (Chen et al., 2013; Chen and Storey, 2014), and presented an overview of dynamic gene expression over aestivation-arousal cycles, identifying candidate genes and pathways involved in metabolism, antioxidant protection, immune defense, protein synthesis and substance transport that are important to aestivation (Zhao et al., 2014). However, protein variety and abundance can often be different from the corresponding gene expression as assessed by mRNA levels, and differential protein expression probably more directly reflects regulatory changes related to aestivation. Until now, translation-level regulatory mechanisms supporting aestivation have not been documented.

To accomplish this, we employed state of the art proteomic techniques, an isobaric labeling quantitation approach using the tandem mass tags (TMT) method coupled with two-dimensional LC-MS/MS (Thompson et al., 2003). These techniques make it possible to investigate the proteome of model and non-model marine organisms and to characterize proteome-wide changes in response to environmental stressors (Tomanek, 2011). In recent years, similar high-throughput proteomics methods have been successfully applied to study aestivation in freshwater apple snails, *Pomacea canaliculata*, using iTRAQ-coupled two-dimensional LC-MS/MS (Sun et al., 2013) and hibernation in ground squirrels and marmots using label free (Shao et al., 2010), heavy isotope labeling (Rose et al., 2011), and iTRAQ (Li et al., 2013) methods. In the present study, we analyzed the global proteomic profile of the sea cucumber *A. japonicus* in aestivation, aiming to understand the role of proteome regulation during aestivation-arousal cycles and determine whether similar molecular responses by the proteome occur in aquatic aestivators as compared with other forms of hypometabolism.

## 2. Materials and methods

### 2.1. Animals

Adult *A. japonicus* (70–108 g body weight) were collected from wild ponds in Jiaozhou Bay of the Yellow Sea in China. Three distinct phases of animals were sampled during the active-aestivation-arousal cycle: (1) Non-aestivation sea cucumbers (NA) as the control group (sea water temperature about 15 °C). These animals had gone through the aestivation period and returned fully to active status (about 6 months after aestivation); (2) Deep-aestivation (DA) (sea water temperature above 25 °C). These animals were sampled after about 15 days of continuous aestivation as indicated by cessation of feeding and locomotion, and the degeneration of the intestine into a very tiny string (about 2 mm); (3) Arousal from aestivation (AA) (sea water temperature about 18 °C). These animals were recently aroused from aestivation (after 2 days back to 18 °C) and moving and feeding were observed. The intestine from 9 sea cucumbers (3 treatments × 3 biological replicates) were dissected without contents and immediately frozen in liquid nitrogen, then kept at −80 °C until subsequent analysis. No permission was needed for sea cucumber collection; the sea cucumber (*A. japonicus*) is not an endangered or protected species.

### 2.2. Protein extraction and trypsin digestion

Intestine samples (~100 mg each) were ground to a powder under liquid nitrogen and homogenized using a Polytron in 1 ml lysis buffer containing 8 M urea, 1% Triton-100, 65 mM dithiothreitol (DTT), with protease inhibitor Cocktail Set IV added. A mild sonication was applied three times on ice to further break the homogenates using a high intensity ultrasonic processor (Scientz). The samples were then centrifuged at 4 °C and 20,000 g for 10 min. Supernatants containing soluble proteins were collected and proteins were precipitated using cold 15% w:v trichloroacetic acid (TCA) for 2 h at −20 °C. Precipitated proteins were pelleted at 4 °C and 5000 g for 10 min and the supernatant was discarded. The pellets were washed three times with 1 ml cold acetone, then re-suspended in rehydration buffer (8 M urea, 100 mM TEAB buffer, pH 8.0) and protein concentration was quantified using the 2D Quant kit (GE Healthcare, Piscataway, NJ). Afterwards, trypsin digestion was carried out overnight at 37 °C with a 1:50 w:w ratio of trypsin to protein. DTT was then added to a final concentration of 5 mM followed by incubation at 50 °C for 30 min. After that, 20 mM iodoacetamine was added to alkylate proteins followed by incubation at room temperature (RT) in the dark for 45 min, then quenching with 30 mM cysteine at RT for 30 min. Trypsin digestion was conducted again with a 1:100 w:w ratio at 37 °C for 4 h.

### 2.3. TMT labeling

Six-plex TMT labeling (Thermo Scientific) was performed according to the manufacturer's instructions with minor revision to isobarically label primary amino groups and thus allows simultaneous comparison of six samples. Briefly, one unit of TMT reagent (defined as the amount of reagent required to label 100 μg of protein) was reconstituted in 24 μl acetonitrile (ACN). The peptide digest from each sea cucumber condition was reconstituted in 0.5 M TEAB, mixed with the TMT reagent and incubated at RT for 1 h. Peptides derived from the control (NA), deep aestivation (DA), and arousal from aestivation (AA) treatments were labeled with the isobaric tags 126, 127, 128, 129, 130 and 131, respectively, following the manufacturer's protocol. After a 2 h labeling at RT, samples from the three treatments were desalted using a Strata X C18 SPE column (Phenomenex) and dried by vacuum centrifugation. A total of 9 samples were labeled using two runs (a 6-plex and a 3-plex). Sample labeling information is listed in Table 1. For each labeling experiment, reporter ions intensity was normalized with average. Normalized reporter ions intensity represents relative quantitation of each sample, which avoids bias of loading quantity in different labeling experiment. Our comparison results are highly consistent among three replicates (Fig. S1).

### 2.4. HPLC fractionation and LC-MS/MS analysis

Each dried peptide sample was fractionated by high pH reverse-phase HPLC coupled with an Agilent 300 Extend C18 column (5 μm particles, 4.6 mm ID, 250 mm length). Briefly, a gradient from 2% to 60% ACN in 10 mM ammonium bicarbonate (pH = 8) was applied to fractionate the peptides into 80 fractions over 80 min. The peptides were then combined into 18 fractions and dried by vacuum centrifugation.

**Table 1**
Labeling information of TMT reporter tags.

| RUN | Treatment | Sample ID | Labels |
|---|---|---|---|
| 1 | NA | 18C | 126 |
| 1 | NA | 20C | 127 |
| 1 | DA | 77C | 128 |
| 1 | DA | 78C | 129 |
| 1 | AA | 141C | 130 |
| 1 | AA | 142C | 131 |
| 2 | NA | 22C | 129 |
| 2 | DA | 79C | 130 |
| 2 | AA | 146C | 131 |

Note: NA: non-aestivation; DA: deep-aestivation; AA: arousal from aestivation.