Original paper

Distribution and Chemoenzymatic Removal of Heavy Metals in Sea Cucumber *Acaudina leucoprocta*

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Sea cucumbers are traditional marine food in Asian countries. In this work, the chemical elements accumulated in sea cucumber *Acaudina leucoprocta* from the East China Sea were measured and analyzed. The contents of Zn, Mn, Cu, As, Pb, Cd and Cr increased with the increase of weight of *A. leucoprocta*. The levels of Pb and As in all the examined individuals were higher than the maximum residue limits (MRLs) permitted in foodstuffs. The metals were found to be selectively distributed in the body compartments of *A. leucoprocta*. The highest contents of heavy metals As, Pb were found in intestines and body wall, while the lowest in anus and internal organ. An efficient chemoenzymic method by combining proteolytic enzymatic hydrolysis with citric acid soaking was established to remove the heavy metals in sea cucumber *A. leucoprocta*. The concentrations of both As and Pb were decreased to below 0.5 mg kg⁻¹ in all the samples.

Keywords: distribution, heavy metal, sea cucumber, *Acaudina leucoprocta*

Introduction

Sea cucumbers, traditional marine food in Asian countries, are widely exploited due to their relatively high nutritional and medicinal values (Hu et al., 2010). Epidemiological studies indicated that sea cucumbers are useful in treatment of stomach ulcers, wound healing, and coronary heart disease (Rahman, 2015). Currently, approximately 1400 species have been identified worldwide. There are more than 20 kinds of edible sea cucumbers in China. In the past decade, the consumption of sea cucumber was increased rapidly (Eriksson and Clarke, 2015; James, 2001).

Sea cucumbers are species of echinoderm with mucous skin, which can potentially accumulate heavy metals by absorption from the polluted sediments in the sea (Luo et al., 2010; Wang et al., 2015; Zhang et al., 2016). The consumption of the sea cucumbers containing high level of heavy metals may potentially cause serious negative impacts on health, since they are persistent, bioaccumulative and toxic (King et al., 2010; Nghia et al., 2009; Noël et al., 2011). Therefore, it is important to ensure that the heavy metals in sea cucumbers which are sold for consumption remain below the maximum residue limits (MRLs) for Food Safety Standards. However, due to the lack of effective methods for removing heavy metals, large amount of sea cucumbers are discarded or processed with very poor quality each year (Eriksson and Clarke, 2015; Gu et al., 2011). *Acaudina leucoprocta*, which has been proven to be a high-quality protein resource, is abundantly distributed in the East China Sea and remains an under-utilized species for consumption due to contamination by heavy metals. The removal of heavy metals is the key bottleneck for the usage of...
sea cucumber *A. leucoprocta* for consumption.

To date, although several studies have reported the distribution of metal elements in sea cucumber *Holothuria tubulosa, Australostichopus mollis* and *Apostichopus japonica* (Culha et al., 2016; Li et al., 2016; Slater and Jeffs, 2010; Warnau et al., 2006), there have been few studies concerning heavy metals removal in *A. leucoprocta*. As an important protein source, it is necessary to assess the heavy metals levels accumulated in *A. leucoprocta*. In this work, the distribution of chemical elements in *A. leucoprocta* was investigated and a chemoenzymatic method for removing the heavy metals in *A. leucoprocta* was developed.

**Materials and Methods**

**Materials**  Sea cucumbers (*A. leucoprocta*) were collected from Xiangshan (Zhejiang, China) (Fig. 1). All the samples were frozen at -20°C. Papain (EC 3.4. 22.2) (800 U mg⁻¹) was purchased from Shanghai Yuanye Bio-Technology Co., Ltd. (Shanghai, China). Pepsin (EC 3.2.3.1) (lyophilized powder, 683 U mg⁻¹) from porcine gastric mucosa was purchased from Sigma-Aldrich (Shanghai, China). HNO₃ and HClO₄ were of HPLC grade and purchased from local supplies. The standard solutions of metals (1,000 mg L⁻¹) used for calibration were obtained from the National Standards Centre. All other reagents used were of analytical grade and obtained commercially.

**Sample preparation**  The frozen sea cucumbers *A. leucoprocta* were thawed at room temperature and then washed repeatedly with deionized water to remove sediment and other impurities. For determination the chemical elements in each body compartments, the body wall, anus, internal organ and intestines of sea cucumber were separated and then homogenized using a laboratory mill (IKA Ultra-Turrax T25, German), respectively (Fig. 2).

**Determination of heavy metals**  Homogenized samples (0.5 g wet weight) were digested with a Multiwave 3000 oven microwave closed system (Anton Paar GmbH, Austria). The digestion program was performed by employing 4 mL of HNO₃ (14.5 M) and 2 mL of H₂O₂ (11.3 M). The microwave digestions were carried out successively at 120, 150 and 190°C for 5, 5 and 20 min, respectively. After digestion, sample solution were concentrated to 2 mL at 120°C, and diluted to a volume of 25 mL for the determination of heavy metals by Varian 820 ICP-MS (Varian, USA). Internal standard solution containing 1 mg/L of Yttrium (Y), Indium (In) and Scandium (Sc) were added to monitor possible matrix effects and
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The concentrations of each element were calculated from linear calibration plots obtained by measurement of the mass spectrum of standard solutions. The corresponding isotopes used in ICP-MS were $^{65}$Cu, $^{64}$Zn, $^{53}$Mn, $^{206}$Hg, $^{75}$As, $^{208}$Pb, $^{111}$Cd and $^{54}$Cr, respectively. All analyses were carried out in triplicates. Results were calculated as average ± standard deviation.

**Removal of heavy metals** In order to inactivate the autolysis enzymes existing in the sea cucumber, the body wall of sea cucumber was autoclaved at 121°C for 15 min, followed by cooling and flushing with deionized water. The prepared samples were treated by the chemoenzymatic method to remove the heavy metals. The pretreated sample was firstly hydrolyzed in phosphate buffer (0.1 M, pH 7.0) by addition of 0.15% papain and pepsin (1:1, w/w) at 37°C for 45 min, then washed with deionized water and soaked in citric acid solution (0.1 M) with 1:10 (w/v) by shaking for 54 h, the citric acid solution was changed every 24 h. Samples after different treatment were homogenized for determination of As and Pb content.

**Scanning electron microscope (SEM) of the sea cucumber body wall** The body wall of *A. leucoprocta* was treated in phosphate buffer (0.1 M, pH 7.2) by adding 2.5% glutaraldehyde for 24 h, then dehydrated by gradient elute of 50–100% ethanol. The outer layer of the body wall was characterized by a field scanning electron microscope (SEM) on a JEOL JSM-5300. Image processing and analysis of fiber diameter and degree of alignment were performed with Image J (http://rsb.info.nih.gov/ij/). The samples for SEM was collected by drying to constant weight, mounted on metal stubs, and then sputter-coated with gold-palladium for one minute.

**Statistical analysis** All experiments were carried out in triplicates. Data were calculated as average ± standard error and a probability value of <0.05 was considered significant difference. Analysis was performed using R-3.4.2 (www.r-project.org) and Origin 8.0 (OriginLab, USA).

**Results and Discussion**

**Distribution of heavy metals in *A. leucoprocta*** *A. leucoprocta* is widely distributed in China Bohai Sea, Yellow Sea and East China Sea. In order to utilize this abundant aquatic product for consumption, it is necessary to firstly investigate the distribution of heavy metals in *A. leucoprocta*. A great amount of natural and human produced heavy metals such as As, Cu, Pb, Zn, Hg, Cd, Cr were the main pollution source to the sea environment (Chen et al., 2016). Considering that the concentrations of the eight heavy metals (Zn, Mn, Cu, Hg, As, Pb, Cd and Cr) in the mud are relatively higher in the East China Sea. So, we investigated the eight heavy metals distributed in the *A. leucoprocta*, which inhabit in the sea mud. The heavy metals concentrations accumulated in the body wall of 55 sea cucumbers *A. leucoprocta* with different weight were measured and the results were shown in Fig. 3. The accumulated concentrations of heavy metals in the *A. leucoprocta* were found in the following order: Mn>Zn>As>Pb>Cu>Cr>Cd>Hg. The highest concentration of Mn in all samples reached $102.47 \pm 0.26$ mg kg$^{-1}$. The concentration of Zn was in the range of $3.88 \pm 0.08$ to $17.68 \pm 0.12$ mg kg$^{-1}$. The results indicated that *A. leucoprocta* has a tendency to accumulate essential micronutrients such as Mn and Zn. The lowest concentration was observed for Cd as well as Hg, which were lower than the MRLs (0.5 mg kg$^{-1}$). This result confirms that bioaccumulation of Hg in sea cucumber is low (McAloon and Mason, 2003). Compared to Mn and Zn, the concentrations of Cu were one order of magnitude lower. The concentrations of the toxic metal As were in a range of $0.98 \pm 0.04$ to $11.19 \pm 0.08$ mg kg$^{-1}$, suggesting that the As tended to accumulate in the *A. leucoprocta*. In addition, it was also observed that the concentration of As was higher than that of the essential metal Cu. This finding is in agreement with the results for *A. japonicus* from the Bohai and Yellow Seas in northern China (Jiang et al., 2015).

Compared with the heavy metal contents in sea cucumber *H. tubulosa*, the concentrations of Zn, Cu and Pb contained in *A. leucoprocta* were relatively higher, while Cd was lower (Warnau et al., 2006). The concentrations of Zn, As, Pb, Cd and Hg were similar to sea cucumbers products from Guangzhou (Wen and Hu, 2010). Compared with the elements levels in *A. japonicus* juveniles from coastal areas of Bohai and Yellow seas, the concentrations of Cu, Cr, Zn, As and Pb were all higher except for Cd and Hg. Based on the national limits of food legislation for heavy metals in China, the MRLs for Cu, Zn, Cr, Pb, Cd, As and Hg are 50, 30, 2.0, 0.5, 0.5 and 0.5 mg kg$^{-1}$, respectively. In this work, the contents of Cu, Zn, Cr, Cd and Hg in different individuals of *A. leucoprocta* body wall were all lower than the MRLs, while the concentrations of As and Pb exceeded the MRLs.

The bioaccumulation of heavy metals is varied in different species, even in the individuals of the same species due to various factors, including physical differences, habitat, age, and season (Morgano et al., 2011; 2014). As shown in Fig. 3, the concentration of As in the *A. leucoprocta* with an approximate weight of 90 g was $7.85 \pm 0.11$ mg kg$^{-1}$, which is 336% higher than that with a weight of 30 g. Similar results were observed for Zn, Mn, Cu, Pb, Cd and Cr. It can be concluded that the concentrations of these elements in *A. leucoprocta* increased with the increase of weight. However, the concentrations of Hg remained relatively stable for different weight classes. These results indicated that Zn, Mn, As, Cu, Pb, Cd and Cr tended to bioaccumulate as the *A. leucoprocta* continues to growth. Warnau et al. (2006) reported that the body compartment was the predominating factor of variation for many heavy metals within an individual organism. Therefore, in order to further investigate the distribution of heavy metals in sea cucumber, four body compartments (body wall, anus, internal organ and intestines) (Fig.2) of *A. leucoprocta* were separated and homogenized to determine the heavy metal concentrations. As shown in Table 1, the contents of Cu, Zn, Cr, Cd and Hg in the body wall, anus and internal organ of *A. leucoprocta* were all lower than the MRLs.
except As and Pb. The intestines and body wall of *A. leucoprocta* accumulated higher concentration of As and Pb. It was demonstrated that the body wall and intestines were the major compartments polluted by the two heavy metals. These results are similar to those in sea cucumber *Holothuria tubulosa* from the Mediterranean Posidonia oceanica ecosystem (Warnau et al., 2006), yet different from those concerning Cu measurements in *H. leucospilota* and Pb in *A. japonicus* from the Bohai Sea (Warnau et al., 2006; Xing and Chia, 1997). Benthos has a potential to accumulate chemical elements from surrounding water or sediments in excessive amounts (Ca et al., 2006; Shulkin et al., 2003). The sea cucumber *A. leucoprocta* inhabited at the bottom of seafloor, and lived on the nutrition from the mud. The concentration of Pb in the surface sediments of East China Sea was very high (34.13 mg kg⁻¹) (Chen et al., 2016). Our investigation indicated that the heavy metals of As and Pb in sea mud in East China Sea where the sea cucumbers *A. leucoprocta* inhabit were 19.32 ± 1.12 and 29.49 ± 0.94 mg kg⁻¹ respectively. As shown in Fig. 2, the inner layer and outer layer of the body walls as well as the intestines contact directly to the mud, which may result in the accumulation of high levels of heavy metals. These results showed that the higher concentrations of As and Pb in body wall and intestines of *A. leucoprocta* were probably due to the following facts. First, the sea cucumber *A. leucoprocta* lives in the buried mud at the bottom of seafloor. Second, the body wall secretes very sticky mucus, which can easily adsorb heavy metals in the mud. Third, the *A. leucoprocta* swallows polluted sea water and mud to feed itself. Based on the above reasons, heavy metals (As and Pb) severely accumulate in the intestines and body walls of *A. leucoprocta*.

**Marketing survey on the residual of heavy metals contained in the *A. leucoprocta* A** marketing survey was performed in order to better understand the residual levels of harmful heavy metals in *A. leucoprocta* consumed in the local market. In general, there are three methods for removing the heavy metals accumulated in the body wall of *A. leucoprocta*: (1) citric acid (0.1 M) soaking for 48 h, (2) NaOH (0.5%) soaking for 12 h, (3) EDTA (5%) soaking for 48 h (Lin et al., 2016). A total of 29 samples, which were treated by different processing methods, were collected from the local markets in Xiangshan. The appearances of *A. leucoprocta* after being treated with the different methods are shown in Fig. 4. The appearance of

### Table 1. Concentrations of elements in different compartments of the sea cucumber *A. leucoprocta* from East China Sea.

<table>
<thead>
<tr>
<th>Samples</th>
<th>Concentration of chemical elements (mg kg⁻¹)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Cu</td>
</tr>
<tr>
<td>body wall</td>
<td>2.13 ± 0.01</td>
</tr>
<tr>
<td>anus</td>
<td>0.75 ± 0.06</td>
</tr>
<tr>
<td>internal organ</td>
<td>1.43 ± 0.07</td>
</tr>
<tr>
<td>intestines</td>
<td>7.16 ± 0.34</td>
</tr>
</tbody>
</table>
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Fresh sea cucumber A. leucoprocta was rust red due to the accumulation of high concentrations of metal elements on its body wall. When the body wall of A. leucoprocta was treated by citric acid soaking, NaOH soaking and EDTA soaking, the appearances were turned to gray, brown and canary yellow, respectively. The residual levels of five heavy metals Hg, As, Pb, Cd and Cr in all samples were determined and analyzed. The results showed that the contents of Cd, Cr and Hg in all of the 29 samples were lower than the MRLs, while the concentrations of As (17 in 29 samples) and Pb (16 in 29 samples) exceeded the MRLs. Both As and Pb exceeded the MRLs in 14 samples. The average concentrations of As treated by three methods (0.58 ± 0.35, 1.58 ± 2.06, 0.64 ± 0.45 mg kg\(^{-1}\) respectively) were all exceeded the MRLs and the concentration of Pb treated by citric acid and NaOH solution (0.55 ± 0.13 and 1.17 ± 1.08 mg kg\(^{-1}\), respectively) were both higher than the MRLs. The citric acid and EDTA soaking methods were more efficient for removing As and Pb than NaOH method. However, the concentrations of As or Pb in some processed samples still exceeded the MRLs. The levels of As and Pb in the sea cucumbers treated by citric acid method (4 in 8 samples) were higher than the MRLs. The residual concentrations of As for the four samples were in the range of 0.53 ± 0.06 to 1.33 ± 0.07 mg kg\(^{-1}\), while Pb were in the range of 0.58 ± 0.04 to 0.75 ± 0.06 mg kg\(^{-1}\). The sea cucumbers treated by NaOH soaking have residual concentration of As greater than MRLs (11 in 14) and Pb (10 in 14). The residual concentrations of As for the 11 samples were in the range of 0.53 ± 0.02 to 6.62 ± 0.16 mg kg\(^{-1}\), while Pb were in the range of 0.61 ± 0.03 to 3.49 ± 0.12 mg kg\(^{-1}\). Two of the 7 samples of sea cucumbers treated by EDTA soaking exceeded the MRLs for As and Pb. The residual concentrations of As for the two samples were 1.27 ± 0.13 and 1.30 ± 0.16 mg kg\(^{-1}\), while Pb were 0.57 ± 0.04 and 0.67 ± 0.07 mg kg\(^{-1}\), respectively.

**Removal of heavy metals in the body wall of A.leucoprocta**

Based on the above results, the concentrations of As and Pb in the body wall exceeded the MRLs. Therefore, it is necessary to establish an efficient and stable processing method to remove the As and Pb from A. leucoprocta to ensure safety for the consumption of sea cucumbers. The body wall of sea cucumber secretes a mucus protein which adsorbs heavy metals from mud, and forms an impermeable structure, making chemical reagents difficult to chelate the heavy metals in both the inner and outer layers of the body wall. It may be feasible to hydrolyze the mucus protein with protease before removing heavy metals by chemical reagents, which loosened and created breaks in the body wall structure. EDTA and NaOH are not suggested for the treatment of the A. leucoprocta. Organic acids are commonly used as food additives. The method combing enzyme hydrolysis with acid soaking may be efficient for the removal of heavy metals. In this study, papain and pepsin were used as enzyme complex to hydrolyze the mucus protein of body wall with the addition of 0.15% (1:1, w/w) at 37°C for 45 min, followed by soaking in citric acid with 0.1 M for 54 h to remove the heavy metals. The resulting A. leucoprocta showed black appearance (Fig.4 B). The structure of the body wall was analyzed by scanning electron microscope. As shown in Fig. 5, compared with sample without treatment by papain and pepsin, the body wall after hydrolysis treatment by enzyme complex was looser. Fig. 6 showed that more than 90% of the As was removed after soaking for 30 h, while Pb required 42 h. The removal efficiencies of As and Pb were both enhanced to 98.22 ± 0.91 % and 94.11 ± 1.08% after soaking for 54 h. The
heavy metals were likely to be removed more thoroughly by the combination of a composite proteolytic enzymatic hydrolysis followed by acid soaking. In order to evaluate the stability of the processing method, twenty-six fresh sea cucumbers of *A. leucoprocta* were treated by the above method. The concentrations of both As and Pb were decreased to below 0.5 mg kg\(^{-1}\) in all samples. The highest residual concentration of Pb in the sample was decreased to 0.17 ± 0.01 mg kg\(^{-1}\), which was almost one-third of MRLs. The highest residual concentration of As was 0.49 ± 0.01 mg kg\(^{-1}\), and is also lower than the MRLs. This result shows that the developed processing method worked well. Sea cucumber *A. leucoprocta* treated by the above method meets the safety standards for commercial requirements. As shown in Fig. 7, all the four treating methods have the ability for reducing the heavy metals from the sea cucumber body walls. The average concentrations of heavy metals (As and Pb) after each treating solvents were significantly lower than their level in fresh sea cucumbers. Kruskal-Wallis one-way analysis of variance was carried out to check the differences between methods for removing heavy metals of As and Pb (data not shown). Compared with the samples without treatment, sea cucumbers treated by citric acid soaking, NaOH soaking, EDTA soaking and chemoenzymatic methods were significant difference (\(p < 0.05\)) for heavy metals As and Pb. The chemoenzymatic method was significant difference compared with the other three chemical methods for the removal of heavy metals of As and Pb.

**Conclusions**

The sea cucumber *A. leucoprocta*, is commonly grown at the bottom of the East China Sea. The *A. leucoprocta* body wall is vulnerable to absorption and accumulation of heavy metals, which may be considered as a valuable bioindicator for evaluating and monitoring metals pollution in seawater. In this study, we investigated the distribution of eight heavy metals (Cu, Zn, Mn, Pb, As, Cd, Cr and Hg) in different individuals and body compartments of *A. leucoprocta*. The results indicated that the levels of Cd and Hg in *A. leucoprocta* were both very low (<0.23 mg kg\(^{-1}\)), while the levels of As and Pb were higher than the MRLs in China. The Mn, Zn, As and Pb tended to accumulate continuously with increasing weight of an individual, while this tendency was not observed for Hg. Due to the characteristics of secreting protein mucus and swallowing mud/seawater, the heavy metals of As, Pb in *A. leucoprocta* were mainly distributed in body wall and intestines. Addition of papain and pepsin in the processing method led to a better loosening and unfolding of the protein backbone. Both As and Pb concentrations were decreased sharply by treatment with papain/pepsin composite and citric acid soaking. This work provides a potential method for removal of heavy metals, laying a foundation for the development and utilization of *A. leucoprocta* for consumption.

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