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Characteristics of minerals extracted from the mid-gut gland of Japanese scallop *Patinopecten yessoensis* at various pH values

Joko Santoso · Yuka Ishizuka · Yumiko Yoshie-Stark

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Abstract This experiment was carried out to determine the proximate composition of the mid-gut gland (MGG) of the Japanese scallop *Patinopecten yessoensis* to evaluate the characteristics of divalent minerals and water-soluble protein at various pHs, and to examine Cd-binding protein at different molecular weights. MGG of scallop contained protein, fat and ash of 28.9 g/100 g dry matter, 44.6 g/100 g dry matter and 6.78 g/100 dry matter, respectively. MGG also contained the macrominerals sodium, potassium, magnesium and calcium, and the trace minerals iron, zinc, cadmium and copper. The solubility of divalent minerals and water-soluble protein was high in both acidic and alkaline conditions, except that magnesium was not affected by acidity. The solubility of copper and iron had a positive correlation with water-soluble protein at all pH values, whereas cadmium had a strong correlation at alkaline pH. Low-molecular-weight water-soluble protein (fraction III, $437.5 < MW < 1{,}355$) bound cadmium strongly in acidic, neutral and alkaline conditions. However, in acidic conditions cadmium had the strongest binding to protein.

J. Santoso
Department of Aquatic Products Technology, Faculty of Fisheries and Marine Science, Bogor Agricultural University, Campus IPB Dramaga, Bogor 16680, Indonesia

Y. Ishizuka
Department of Food Science and Technology, Faculty of Marine Science, Tokyo University of Marine Science and Technology, Konan Minato-ku, Tokyo 108-8477, Japan

Y. Yoshie-Stark (✉)
Department of Food Life Science, Faculty of Life Science, Toyo University, Itakura-machi Ora-gun, Gunma 374-0193, Japan
e-mail: yumiko_y@toyo.jp

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Introduction

The scallop is an edible bivalve that has been extensively cultivated in the sea at the northern part of Japan. Before the earthquake and tsunami struck the northeastern part of Japan, more than 500,000 tons of scallops per year were produced in 2006–2008 [1]. The edible part of the scallop is an adductor muscle, which is shipped to the market for consumption. The other part, namely the mid-gut gland (MGG), which is called "uro" in Japanese, is left at fishing sites as waste. The waste contains large amounts of high-quality protein, amino acids, fats and fatty acids such as eicosapentaenoic and docosahexanoic acid, as well as minerals like calcium, magnesium, sodium and phosphorus [2]. The use of biomass and organic wastes as potential sources of materials and energy has recently increased because of the production of new valuable compounds and new environmental policies [3]. Therefore, the MGGs of scallops may become a valuable resource.

Cephalopods including the scallop are known to accumulate high levels of essential and non-essential elements as they feed by filtering particles from the water [4–8]. In scallops, harmful metal ions are concentrated in the whole individual internal organs, especially in the digestive gland and kidney [8–10]. Cadmium is one of the harmful metal ions that can accumulate in the mid-gut gland and kidney. In Japanese scallops, for example, the concentrations of cadmium in the mid-gut gland were 20–40 mg/l [2] and 13.7 mg/l [11] depending on the water conditions where they lived. Besides this, the digestive gland and kidney also

contain useful compounds, e.g., minerals such as sodium, calcium, magnesium, iron [2], peptides [12], protein [8] and peptidase enzymes [13]. Therefore, it is possible to take advantage of the favorable components by eliminating those that are harmful, especially cadmium.

There have been many attempts to reduce the cadmium content from the MGG of scallops using physical, chemical or both treatments [2, 3, 11, 14]. However, the treatment given might also affect other components that are actually beneficial to the organism, especially minerals that have a valence equal to cadmium, as well as protein. Therefore, the most important stage before providing treatment is to determine the chemical profile inside the mid-gut gland of scallops and to understand the behavior of the minerals related to their solubility.

Several studies have revealed that the content, solubility and bioaccessibility of minerals are influenced by several factors, such as pH [15], the presence of other compounds such as a chelating agent, which can be an enhancer or an inhibitor [16, 17], and treatments such as heating or cooking [15, 18, 19]. The first objective of our work was to determine the proximate and mineral composition of the MGG of the Japanese scallop *Patinopecten yessoensis* and to evaluate the properties of minerals in different pH conditions, focusing on their solubility.

Differnt opinions regarding the toxic mechanism of cadmium among researchers worldwide reflects the great complexity of this mechanism. One of the mechanisms is that cadmium has an affinity for the protein-thiol group; thus, it can displace Zn^{2+} and Ca^{2+} from metal-binding protein, thus inhibiting its activities [6, 20–22]. However, proteins with low, intermediate or high molecular weights have also been found as potential binding sites for trace metals [17, 23]. Therefore, the second objective of this experiment was to evaluate the associations between cadmium and protein fractions (different molecular weight) extracted from the MGG of the Japanese scallop at acidic, neutral and alkaline conditions.

Materials and methods

Chemicals and reagents

Chemicals and reagents used in this experiment (nitric acid, standard mineral solutions, hydrochloric acid, sodium chloride and sodium hydroxide) were analytical grade and obtained from Wako Pure Chemical Industries, Ltd., (Osaka, Japan). Bio-Rad protein assay kit was obtained from Bio-Rad (Rockford, IL, USA). Bovine serum albumin was obtained from Sigma-Aldrich Japan Co. (Tokyo, Japan). Sephadex G25 (26 × 300 mm) was obtained from GE Healthcare Biosciences (Pittsburgh, PA, USA).

Japanese scallops and mid-gut gland samples

Japanese scallops *Patinopecten yessoensis* with a shell length of 12 cm were harvested in June 2008. They were obtained from the cultivation farmers in Aomori Prefecture (Mutsu Bay, Yokohama Town), Japan. The sea water temperature of the cultivation/harvested area was 13.0–15.0 °C in June. MGGs were taken from fresh scallops. They were washed with tap water, wiped with paper towels, minced in a food processor (MK-K75, Matsushita Electric, Co., Osaka, Japan) and stored at −30 °C until use.

Proximate analysis

The chemical composition, i.e., moisture, ash, fat and protein (nitrogen converting factor = 6.25), of scallop MGGs was analyzed according to the method of AOAC [24].

Total mineral analysis

Samples (scallop MGG) were treated by the wet ashing method using nitric acid. The procedure consists of the following steps: (1) MGG samples (2 g) were weighed in a flask and 5 ml of nitric acid added. (2) The sample was destroyed using the ETHOS-1 Microwave Digestion System (Milestone General K.K, Shelton, CT, USA). (3) The sample was dissolved in 10 % hydrochloric acid and transferred into a 25-ml volumetric flask. (4) Then it was analyzed using an atomic absorption spectrophotometer (Model AA-660, Shimadzu Co., Kyoto, Japan) with an acetylene flame, a single slot head and a Pt-Rh corrosion-resistant nebulizer for measuring the total sodium (Na, detection limit; 0.005 ppm), potassium (K, detection limit; 0.005 ppm), magnesium (Mg, detection limit; 0.001 ppm), calcium (Ca, detection limit; 0.04 ppm), iron (Fe, detection limit; 0.06 ppm), zinc (Zn, detection limit; 0.05 ppm), cadmium (Cd, detection limit; 0.007 ppm) and copper (Cu, detection limit; 0.04 ppm). Standard solutions were prepared in 10 % hydrochloric acid. All the glassware and plastic bottles used were dipped in Contaminon (Wako Pure Chemical Industries, Ltd., Osaka, Japan) for at least 2 h and then rinsed with double-distilled de-ionized water to remove contaminants.

Determination of soluble mineral and water soluble protein

Prior to obtaining soluble mineral and water soluble protein fractions, MGG samples were treated as follows. Fresh MGG samples (1 g) were blended in a tube with 30 ml of hydrochloric acid, pH 2–5, or milli-Q water or sodium hydroxide, pH 7–12, at 5,000–10,000 rpm for 3 min using

Ultra-Turrax (T-25, Janke and Kunkel, IKA-Labortechniik GmbH Co., Staufen Germany) to obtain water-soluble fractions. The samples were adjusted to pH values from 2 to 12. Next, the samples were centrifuged at 5,500×*g*, 4 °C for 10 min (Kubota 6800, Kubota Corp., Fujioka, Japan). Collected supernatants were de-oiled using chloroform, then the de-oiled layer (water fraction) was measured for the total contents of both soluble minerals and protein. Total mineral contents were measured using an atomic absorption spectrophotometer (Model AA-660, Shimadzu Co., Kyoto, Japan). The solubility of each mineral was calculated using the equation: Solubility (%) = (Soluble mineral mg/g)/(Total mineral mg/g) × 100. Soluble protein contents in each fraction were determined according to the Bradford protein assay (Bio-Rad Protein Assay Kit, Rockford, IL, USA). The resulting absorbance was measured by a spectrophotometer (UV-1200 UV-VIS Spectrophotometer, Shimadzu, Kyoto, Japan) at a wavelength of 595 nm. Bovine serum albumin was used as a standard. The solubility of each water-soluble protein was calculated using the formula: Solubility (%) = (Water soluble protein g/100 g)/(Total protein g/100 g) × 100.

Determination of soluble cadmium in the water-soluble protein fraction using size-exclusion column chromatography

To obtain the dissolved cadmium binding to proteins at different molecular weights, the fractionation process using size-exclusion column chromatography was performed. Briefly, wet MGG samples (1 g) were blended in a tube with 30 ml of hydrochloric acid (pH 2), milli-Q water (pH 6) or sodium hydroxide (pH 12) at 5,000–10,000 rpm for 3 min using Ultra-Turrax (T-25, Janke and Kunkel, IKA-Labortechniik GmbH Co., Staufen, Germany) to produce the water-soluble fraction. The samples were centrifuged at 5,500×*g*, 4 °C for 10 min (Kubota 6800, Kubota Corp., Fujioka, Japan). Collected supernatants were de-fatted using chloroform. The water layer was then evaporated using a speed vacuum to remove the remaining chloroform. Collected samples were eluted to the size-exclusion column chromatography to an appropriate concentration with the following conditions:

- Detector: UV-VIS Detector SPD-10Avp (Shimadzu, Kyoto, Japan)
- Pump: LC-10ADvp (Shimadzu, Kyoto, Japan)
- Temperature: room temperature
- Wavelength: 280 nm
- Column: Sephadex G25 (26 × 300 mm)
- Mobile phase: 50 mM sodium phosphate buffer + 50 mM NaCl (pH 7.0)
- Flow rate: 0.65 ml/min
- Fraction collector: DC 1200 (Tokyo Rikakikai Co., Ltd., Tokyo, Japan)

For the molecular weight marker, three types of substances were used, i.e., Hip-His-Leu peptide (MW = 437.5, Sigma-Aldrich Corp., St. Louis, MO, USA), vitamin B_{12} (MW = 1,355, Wako Pure Chemical Industries Ltd., Osaka, Japan) and ribonuclease A (MW = 13,700, GE Healthcare Biosciences, Pittsburgh, PA, USA). Five fractions were collected after elution, namely fraction I (MW = 13,000), fraction II (MW = 1,355), fraction III (437.5 < MW < 1,355), fraction IV (MW = 437.5) and fraction V (MW <437.5). Collected fractions (I–V) were concentrated by speed vacuum to have appropriate concentrations, and the cadmium contents of each fraction then were measured using an atomic absorption spectrophotometer (Model AA-660, Shimadzu Co., Kyoto, Japan).

Statistical analysis

Results are expressed as mean value ± standard deviation. Comparison of means using a significant level of $p < 0.05$ was performed by analysis of variance and means separated by *F* test and Student's *t* test using SPSS version 16 software.

Results

Proximate and minerals composition

Table 1 shows the proximate composition and mineral profile of scallop MGG. The sample had protein, fat and ash contents of 7.67/100 g fresh weight, 11.9 g/100 fresh weigh and 1.80/100 g fresh weight, respectively. After converting the 100 dry matter samples, the contents of protein, fat and ash were shown to be high, with values of 28.9/100 g dry matter, 44.6/100 g dry matter and 6.78 g/100 dry matter, respectively. Sodium was found to be a major macromineral in scallop MGG (25.44 mg/g dry matter), followed by potassium (8.21 mg/g dry matter), magnesium (3.66 mg/g dry matter) and calcium (0.07 mg/g dry matter). The profiles of trace minerals in order from the highest concentration were iron, cadmium, zinc and copper with values of 255 μg/g dry matter, 160 μg/g dry matter, 115 μg/g dry matter and 71.1 μg/g dry matter, respectively.

Solubility of divalent mineral and water-soluble protein at various pHs

Since the MGG of scallop contained both useful and harmful minerals, it is necessary to study their solubility at different pH values in order to obtain useful information

for utilizing the MGG of scallop safely. The solubility of divalent minerals and water-soluble protein is shown in Fig. 1. The solubility of water-soluble protein and the divalent minerals copper, zinc, iron and cadmium had the same pattern, except for magnesium. Both at high and low pH values, water-soluble protein, and the minerals copper, zinc and cadmium had high solubility; however, iron tended to be more soluble at high pH, and there was no pattern for the solubility of magnesium. The highest solubility of copper, iron and water-soluble protein was found at pH 12, with values of 99, 84 and 81 %, respectively. For cadmium and iron, the highest solubility was found at pH 3 and pH 2, which were 58 and 89 %, respectively. Moreover, at pH 12, the solubility of iron was also high (70 %) and was not significantly different compared to pH 2.

Table 1 Proximate composition and mineral contents of the mid-gut gland of Japanese scallop *P. yessoensis* (mean ± SD, $n = 3$)

Proximate compositions (g/100 g fresh weight)	Values
Moisture	73.4 ± 0.35
Crude protein	7.67 ± 0.78
Total fat	11.9 ± 0.18
Ash	1.80 ± 0.03
Carbohydrate (by difference)	5.23 ± 1.19
Mineral contents	**Values**
Na (mg/g dry matter)	25.4 ± 1.67
K (mg/g dry matter)	8.21 ± 0.05
Mg (mg/g dry matter)	3.66 ± 0.17
Ca (mg/g dry matter)	0.07 ± 0.01
Fe (μg/g dry matter)	255 ± 69.6
Zn (μg/g dry matter)	115 ± 14.9
Cd (μg/g dry matter)	160 ± 34.7
Cu (μg/g dry matter)	71.1 ± 5.35

Relationship of the solubility between divalent minerals and cadmium, divalent minerals and water-soluble protein

Since there is competition among divalent minerals in relation to the solubility as well as minerals and protein, the relationship of their solubility needs to be evaluated. Positive correlations of the solubility of divalent minerals and cadmium, and water-soluble protein and cadmium in different pH values are depicted in Fig. 2. Zinc and cadmium had positive solubility relationships ($r = 0.7395$),

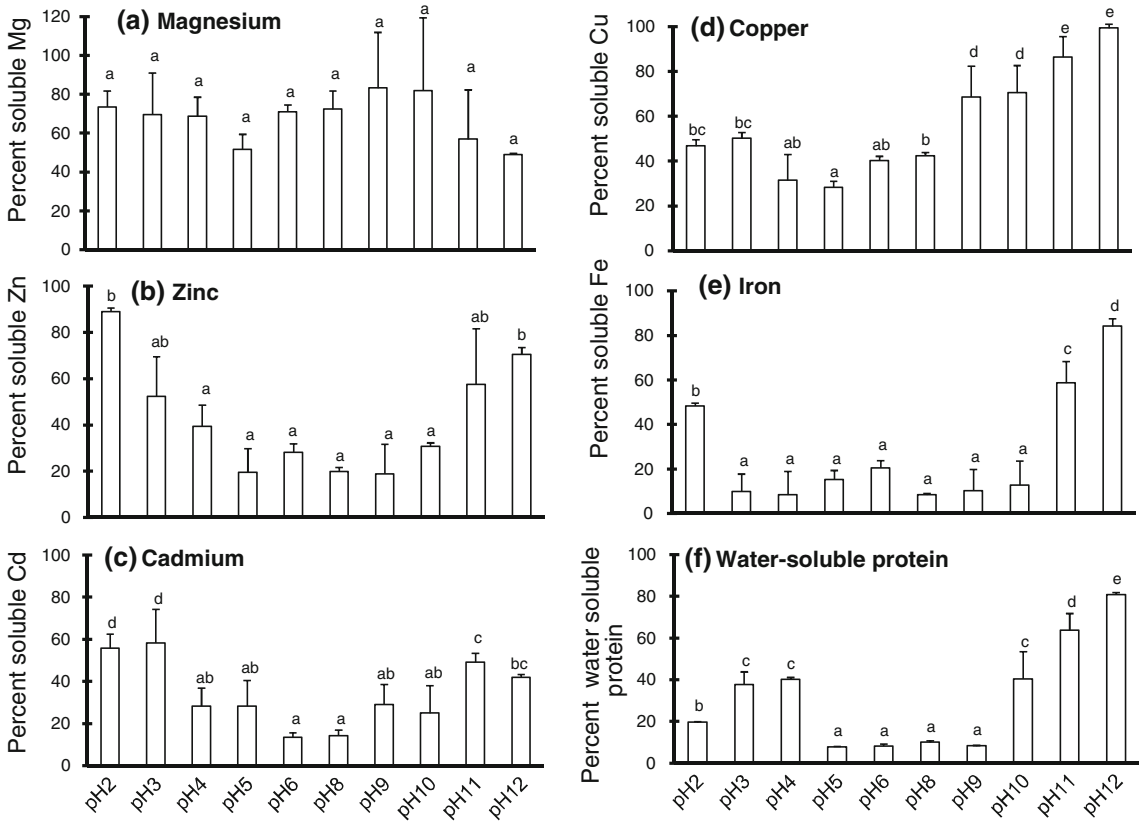

Fig. 1 The solubility of magnesium (**a**), zinc (**b**), cadmium (**c**), copper (**d**), iron (**e**) and water-soluble protein (**f**) extracted from the mid-gut gland of the Japanese scallop *Patinopecten yessoensis* under various pH conditions. *Letters* over each column in the graph not sharing the same are significantly different ($p < 0.05$) (mean ± SD, $n = 3$)

whereas cadmium had a weak relationship with the other minerals magnesium, copper and iron of $r = 0.1277$, 0.4678 and 0.3162, respectively (data not shown).

The positive correlation of the solubility between divalent minerals and water-soluble protein at different pH values was found in the minerals copper ($r = 0.6951$), iron ($r = 0.7265$) and cadmium ($r = 0.4406$). For cadmium, the strong relationship occurred when the pH was alkaline ($r = 0.7128$).

Cadmium-binding water–soluble protein at different molecular weights

In order to understand Cd-binding protein at different molecular weights, we performed fractionation using Sephadex G25 size-exclusion column chromatography. Cd-binding protein had the same pattern at pH 2, 6 and 12; the highest rate of binding cadmium was found for low-molecular-weight protein between 437.5 and 1,355 (fraction III) with values of 0.810, 0.385 and 0.312 μg/g, respectively (Fig. 3, left), or was equal to 35.0, 39.6 and 27.1 %, respectively (Fig. 3, light). Among three pH conditions, protein in acidic conditions could bind cadmium at a total fraction of 2.31 μg/g, followed by alkaline (1.15 μg/g) and neutral conditions (0.937 μg/g).

Discussion

Proximate and mineral composition

For mineral comparison, Metian et al. [7] analyzed the metal content of the digestive gland of the tropical scallop *Comptopalliium radula* in two stations southwest of New

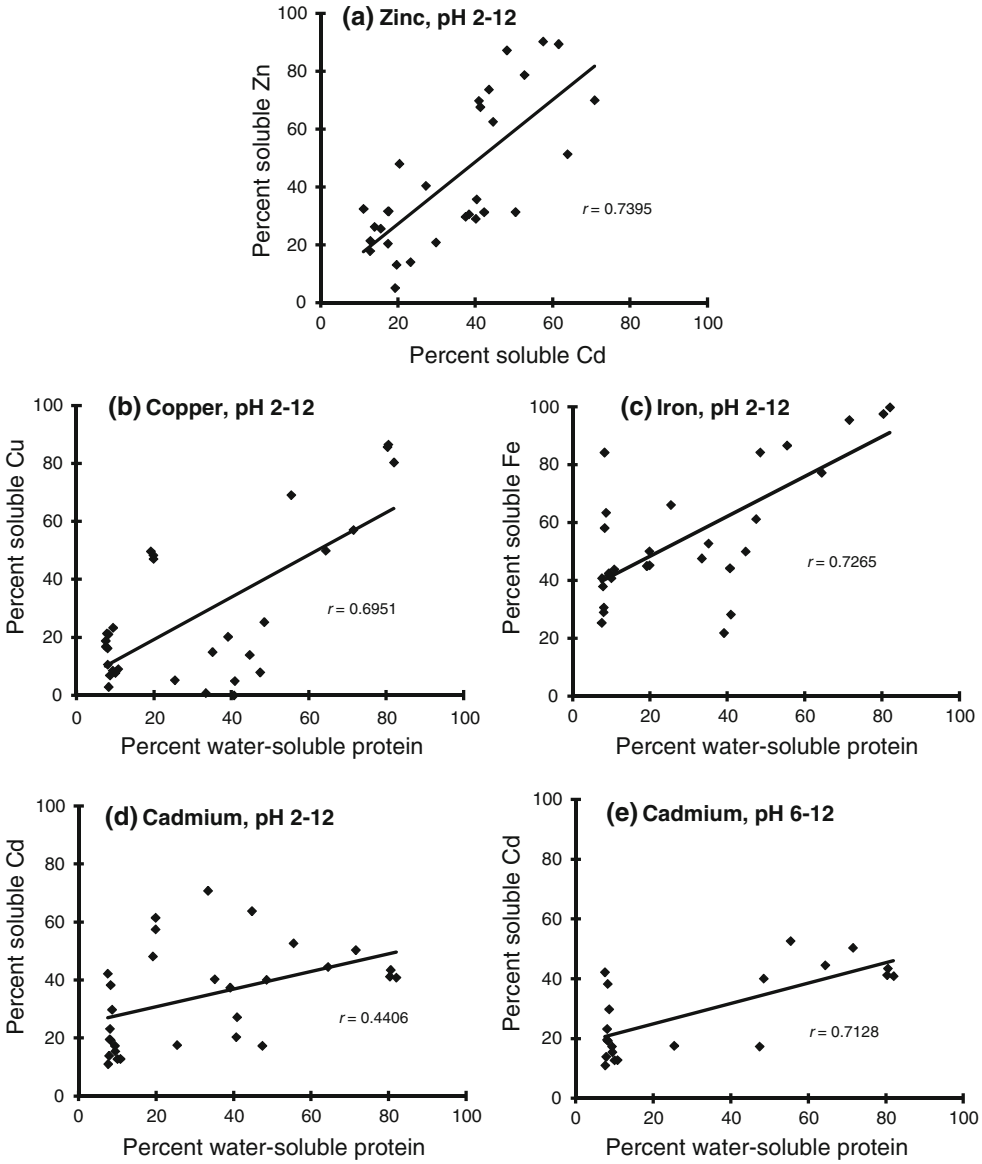

Fig. 2 Positive relationship between the solubility of cadmium and zinc (**a**), copper and water-soluble protein (**b**), iron and water-soluble protein (**c**), cadmium and water-soluble protein at pH 2–12 (**d**) and cadmium and water-soluble protein at pH 2–30 (**e**), extracted from the mid-gut gland of the Japanese scallop *Patinopecten yessoensis* ($n = 16$–30)

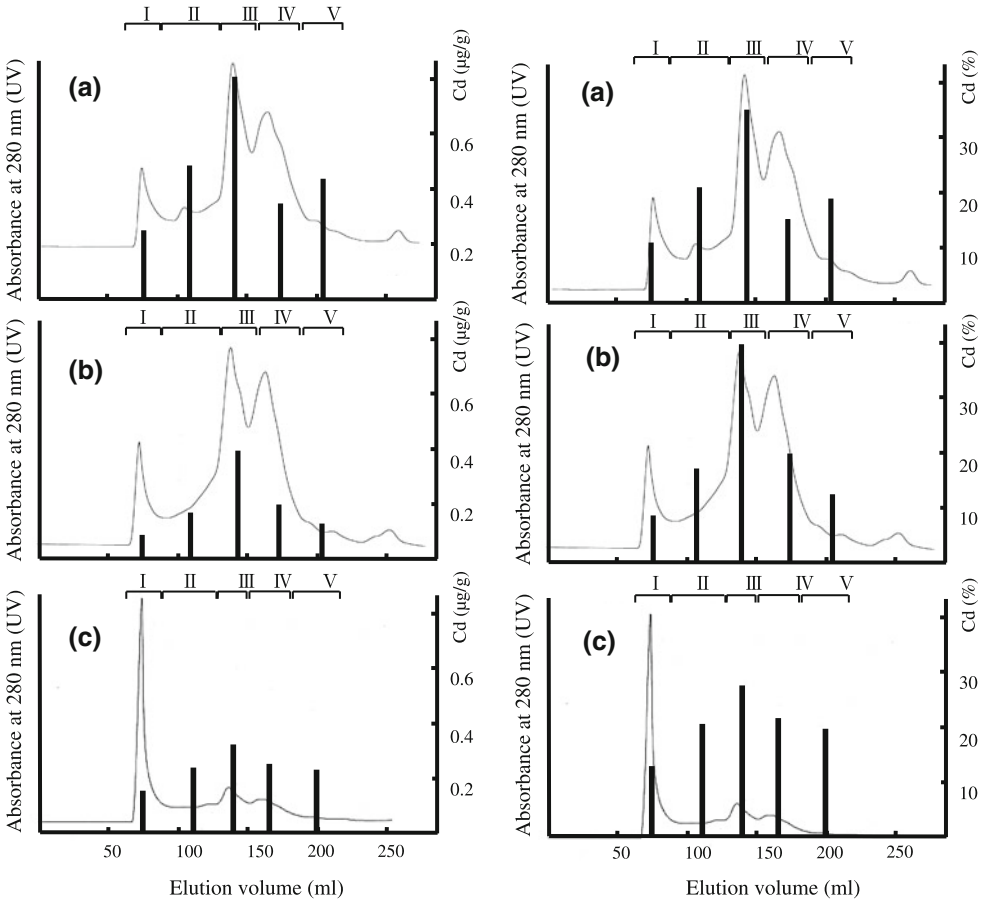

Fig. 3 Size-exclusion column chromatography of the extract of the Japanese scallop *Patinopecten yessoensis* at pH 2 (**a**), pH 6 (**b**) and pH 12 (**c**) on a Sephadex G25 column. *Left* Calculated cadmium at each fraction (μg/g). *Light* Calculated in percent cadmium of each fraction

Caledonia Lagoon. Among four trace minerals, namely iron, cadmium, zinc and copper, iron was determined at the highest concentration of 650–1,372 μg/g dry matter, followed by zinc (580–787 μg/g dry matter), copper (9.82–12.6 μg/g dry matter) and cadmium (0.75–7.11 μg/g dry matter). In Japanese scallop waste from Hokkaido, the concentrations of sodium, potassium, magnesium and calcium were found to be 1,920, 2,362, 660 and 1,078 mg/kg fresh weight, whereas zinc and cadmium contents were 45.5 and 34.5 mg/kg fresh weight, respectively [2]. When we converted these data to a dry matter basis, the sodium, potassium, magnesium, calcium, zinc and cadmium contents were 7.68 mg/g, 9.45 mg/g, 2.64 mg/g, 4.31 mg/g, 182 μg/g, and 138 μg/g, respectively. Similar results were also reported by Shiraishi et al. [11]. We also converted their values to a dry matter basis. They reported that the waste of scallop processing also contained the minerals calcium (5.67 mg/g), magnesium (3.95 mg/g), zinc (133 μg/g), cadmium (54.8 μg/g), iron (18.8 μg/g), manganese (7.44 μg/g) and copper (3.56 μg/g). This suggests that the MGGs of scallops, which are a fishery waste product, contain not only toxic metals, but also important minerals and highly nutritional components that could be extracted for further use. Several studies have shown that scallop processing waste also includes some important components such as cysteine-rich polypeptides [12], amino peptidase enzymes [13], and proteins and amino acids [8, 12, 25].

Solubility of divalent minerals and water-soluble proteins at various pHs

There was a tendency for the solubility of the divalent minerals copper, iron, zinc and cadmium to be positively correlated with the solubility of water-soluble proteins at high pH. This indicated that the mineral binding of water-soluble protein might occur through protein-mineral complex formation.

Minerals contained in MGG of scallop might change their chemical form when subjected to different pHs; therefore, they could interact with other compounds inside, especially water-soluble protein. Santoso et al. [15] reported that the solubility of the Mg and Ca in seaweed increased significantly after being boiled in acetic acid solution compared to being boiled in water or salt solution. The same results of organic acid increasing the solubility of minerals were also shown [26, 27]. Soluble minerals were also found in high concentrations in yoghurt, an acid-type food [28].

However, in this work when we treated the samples with hydrochloric acid or sodium hydroxide to adjust the pH, the divalent minerals tended to be soluble in both acidic and alkaline conditions. It can be presumed that the acid and alkaline treatments change the conformation of the minerals to be more soluble. One possible way is to bind with proteins to establish complexes of soluble mineral-protein. In this case water-soluble protein in the MGG of scallops became an enhancer. Clydesdale [29] defined an enhancer as a molecular species in material that forms a compound with minerals that is soluble and can be absorbed by mucosal cells.

Relationship of the solubility of divalent minerals and cadmium, divalent minerals and water-soluble protein

This study suggested that zinc and cadmium had similar solubility properties, as indicated by the same patterns (Fig. 1). Zinc and cadmium had the same characteristics for their solubility and had a positive correlation; this may be related to its function on metallothioneins. Roesjadi [30] has proposed a model for coupled metallothionein induction and rescue target ligands compromised by inappropriate metal binding, with cadmium and zinc playing interchangeable roles. When non-essential metals such as cadmium, mercury or silver enter a cell, there is inevitability competition between them and existing metals such as copper and zinc for intracellular ligands such as metalloproteins [20]. Therefore, cadmium can also displace zinc as well as calcium [21]. Reeves and Chaneyb [31] reported that cadmium bioaccessibility was also related to the ingestion of other minerals such as calcium, zinc and iron.

This indicated that the minerals copper and iron were extracted with water-soluble protein at all pH values, whereas cadmium tended to be extracted with water-soluble protein at high pH. Increasing the pH values could change the conformation form from water soluble to more soluble (Fig. 1). It means that water-soluble protein became an enhancer to make a complex with cadmium by mineral-protein binding, so that the solubility of cadmium was also high. Mineral-binding proteins, namely metallothioneins, associated with the mechanism of metal detoxification in aquatic invertebrates [20, 32]. Metallothioneins are low-molecular-weight proteins that can be induced by free cytosolic metal ions, especially cadmium, copper, zinc and mercury, and are involved in defending against metal toxicity [6, 33, 34].

Cadmium-binding water–soluble protein at different molecular weights

This condition was in line with the percent solubility of cadmium, which was also high in acidic conditions (Fig. 1). Previous studies conducted by Ghimire et al. [2] also achieved the same result, showing that lower pH treatments using astringent persimmon juice were more highly efficient for leaching cadmium from the MGG of scallops.

One of the mechanisms to remove cadmium from the aquatic organism through Cd-binding sites is a low molecular mass protein such as metallothionein [6]. Protein of low, intermediate or high molecular weight has also been found to be a potential binding site for trace metals, including cadmium [23, 33]. In this experiment, cadmium extracted from MGG of scallop was bound by water-soluble protein at different molecular weights from 437.5 to 13,700. However, in low-molecular-weight water-soluble protein (fraction III, $437.5 < MW < 1{,}355$), cadmium was bound strongly. A similar result was also reported by Raimundo et al. [17] showing that cadmium extracted from the digestive gland of *Octopus vulgaris* had a strong association with low-molecular-weight protein (LMW; 11,000–6,000 Da) and a minor association with high-molecular-weight protein (HMW; 144,000–130,000 Da).

From our results it could be concluded that the MGG of Japanese scallop *P. yessoensis* contained highly nutritional compounds, i.e., protein, fat and minerals. The solubility of divalent minerals and water-soluble protein was high in both acidic and alkaline conditions, except that magnesium was not affected by acidity. The solubility of copper and iron had positive correlations with water-soluble protein at all pH values, whereas cadmium had a strong correlation at alkaline pH. Low-molecular-weight water-soluble protein bound cadmium strongly, especially in acidic conditions.

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