Biol. Stud. 2019: 13(1); 61–70 • DOI: https://doi.org/10.30970/sbi.1301.583
www.http://publications.lnu.edu.ua/journals/index.php/biology

UDC 577.352.38:577.64

THE CONTENT OF METALS IN METALLOTHIONEINS OF THE BIVALVE MOLLUSK *UNIO TUMIDUS* DEPENDING ON DIFFERENT *IN SITU* AND *IN VITRO* EXPOSURES

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Khoma V. V. The content of metals in metallothioneins of the bivalve mollusk *Unio tumidus* depending on different *in situ* and *in vitro* exposures. **Studia Biologica**, 2019: 13(1); 61– 70 • DOI: https://doi.org/10.30970/sbi.1301.583

Metallothioneins are low-molecular-weight proteins involved in homeostasis and detoxification of the transition metals. The bivalve mollusks are known to be the most effective filter feeding aquatic organisms that provide the accumulation of toxic metals from the environment. The analytical methods for detection and quantitative determination of metallothioneins in the biological samples must be improved to adjust the correct conditions for detecting their metal composition. The sodium azide is frequently utilized to conserve the Sephadex and prevent the microbial growth. The aim of this study was to select the conditions for isolation of the metallothioneins of the bivalve mollusk that would ensure a stability of their metal-binding forms.We examined the metallothioneins of mollusks from four rural sites in the basin of Dniester River, upstream and downstream the dam of two hydropower plants (small HPP in the Kasperivtsy, the Seret River and micro HPP in the Krasnostavtsy, the Zhvanchyk River. The mollusks from the most polluted site (the reservoir of small HPP) were subjected to depuration in the laboratory during 21 days, and the metallothioneins from their digestive gland were compared in different conditions of elution. Four compositions of the eluent based on 10 mM Tris-HCl buffer, pH 8.0 were applied: without additions; containing 10 mM 2-mercaptoethanol and 5 mM sodium azide; containing 5 mM sodium azide; and containing 10 mM 2-mercaptoethanol. The obtained results indicated the distortion of the chromatographic and spectral features of metallothioneins in the presence of sodium azide in the eluent. The detecting of the metal composition of the metallothioneins demonstrated that they predominantly bind Zn. In field conditions of two HPPS, the highest level of Cu in the digestive gland tissue and in the metallothioneins was detected in the mollusk from the

reservoir of small HPP. The depuration of the specimens caused the elimination of Cu from the metallothioneins. The ratio of Zn and Cd was rather stable in all studied conditions of the elution. The function of detoxification of the metallothioneins regarding Cd and Cu was less revealed in mollusks from the reservoir of small HPP. The results accented a necessity of adjusting of correct conditions of elution in the investigation of the metallothioneins in mollusks.

Keywords: metallothioneins, bivalve mollusks; copper; zinc; cadmium; gel-permeation chromatography

INTRODUCTION

Metallothioneins (MTs) are low-molecular-weight proteins involved in the homeostasis of the endogenous metals and in the detoxification of several toxic metals [5, 9]. These intracellular proteins are characterized by unusually high cysteine content (30%) and lack of the aromatic amino acids. MTs efficiently bind several divalent and monovalent transition metals, mainly Zn, Cu, Cd, Ag, with high affinity by forming characteristic polynuclear metal-thiolate clusters within their structure [3, 5]. Moreover, MTs are among the most abundant intracellular targets for the biologically essential metals, Zn and Cu [18]. Thus, MTs play an important role in providing metal cofactors for apoproteins and regulate gene expression via association with zinc fingers [10].

MT in a Zn-bound state is very stable that makes it an ideal reservoir for intracellular Zn. However, they are highly sensitive to toxic metals: under cellular stress from toxic transition metals, such as cadmium and mercury, the cell increases MT expression [11]. The synthesized protein sequesters these toxic metals buffering them and protects cell damage. The most important example is connecting to the detoxification of Cd, whereas MTs can bind almost all cadmium content within the cells in complexes of low toxicity [11]. Due to this unique property, MTs are considered to be an ideal tool for bioremediation of toxic metal contaminated environments [4, 8]. However, utilizing of the MTs with the purpose of bioremediation is discussed only for the bacteria [4].

Bivalve mollusks are known as the most effective filter feeding aquatic organisms. They accumulate different toxic substances from water, including toxic metals [7, 11]. Therefore, the elucidation of properties of their MTs can provide utilizing of these organisms in the bioremediation of the reservoirs.

Analytical methods for detection and quantitative determination of the MT in biological samples are provided [5, 16]. The main problem in the MTs determining is the oxidation of protein which can occur in the aerobic conditions and is accompanied by a release of metal from the thiolate clusters [5]. Disulphide-bond formation occurs as thionein becomes bound in the high-molecular-weight region and chemical reduction is necessary to restore its normal elution behavior. Mercaptoethanol added to homogenates maintains the reducing conditions normally found in cellular milieu and prevents the oxidation of the MTs during isolation [10, 12]. However, the MTs of the bivalve mollusks are very sensitive to the oxidation due to comparatively high content of copper in their composition [9]. The aim of this study was to select the conditions for the allocation of MTs of the bivalve mollusk that would ensure the stability of their metal-binding forms.

MATERIALS AND METHODS

Bivalve mollusks *Unio tumidus* (Unionidae) (~ 6 years old, 8±1 cm length, and 42±5 g weight) were collected in four rural sites in the basin of Dnister River, upstream and downstream (U and D sites, correspondingly) the dam of two hydropower plants (HPPs). Kasperivtsi small HPP (K groups) is located in the lower part of the Seret River (48°40′ N, 25°50′ E), and Krasnostavtsi micro HPP (Zh groups) is located on the Zhvanchyk River(48°49′ N, 26°23′ E). The bivalves were collected in summer. The investigation of the mollusks from four sites had started in the day of sampling.The applying of different eluents was made with the samples of the mollusks from KU site (reservoir of the small HPP) that were kept in the aquarium during 21 day under the controlled conditions in the aerated water that was renewed each two days.

For isolation of the MTs, tissue samples from five individuals of the experimental group were pooled in aliquot quality (total mass 350 mg), and homogenized in ice-cold 10 mM Tris-HCl buffer, pH 8.0, containing 10 mM 2-mercaptoethanol to prevent the MTs from oxidation and 0.1 mM phenylmethylsulfonyl fluoride to prevent protease activity [5]. EDTA was not added to the buffer to prevent the loss of metals bound to proteins. MTs from the digestive gland were obtained as low weight thermostable proteins by size-exclusion chromatography on Sephadex G-50 [16], as it was described elsewhere [6]. All chemicals were purchased from Sigma Aldrich (St. Louis, USA) and were of the analytical grade or higher. Column calibration was achieved by applying a mixture of standard proteins: chymotrypsinogen (25.8 kDa), myoglobin (17.0 kDa), cytohrome *c* (12.3 kDa), ubiquitin (8.6 kDa), insulin chain B oxidized (3.5 kDa). In the study of the mollusks from four sites, the low weight thermostable proteins were eluted in the medium contained only 10 mM 2-mercaptoethanol. In the experimental exposure with the depuration of mollusks, four compositions of the eluent based on the 10 mM Tris-HCl buffer, pH 8.0 were applied: 1. without additions (C); 2. containing 10 mM 2-mercaptoethanol and 5 mM sodium azide (corresponding to 0.03%) (ME+N); containing 5 mM sodium azide (N); containing 10 mM 2-mercaptoethanol (ME). MTs were identified as low weight thermostable proteins that have high absorbance at 245 nm (peak corresponding to the metal-thiolate clusters) and absence of peak at 280 nm due to the absence of the aromatic residues [13]. In the combined fractions of the peak (10 mL), UV spectra were recorded and the metal content was determined.

The concentration of Cu, Zn and Cd was measured in the samples of the tissue of mollusk digestive gland (250 mg), and pooled eluate of MTs-containing fractions from a digestive gland after the size-exclusion chromatography (10 mL), as it is described elsewhere [17, 6]. A reliability of measurements towards selected elements was assessed by analyzing ERM-CE 278 certified reference material; recoveries of metals were between 90% and 110%. Quality control was assessed by Quality Control Sample for trace metal and method of Standard Addition (www.dentalmercury.com/245_1.pdf). Metal concentration in tissue was expressed as $\mu g \times g^{-1}$ fresh weight (FW), and in MTs, also as $nmol \times g^{-1}$ FW.

Analysis was of thermostable proteins carried out in triplicate for three independent samples, and metal measurements in the tissue were carried out in six specimens, the same as it was applied earlier [6]. The results were expressed as mean ± SD. Shapiro-Wilk test was used for the assessment of normality. Data were analyzed with parametric Student's *t*-test significant at $p < 0.05$. Statistical calculations were performed with Statistica v 8.0 and Excel for Windows-2000.

RESULTS AND DISCUSSION

Gel-filtration of the thermostable extract from a digestive gland in each experimental group revealed the peak that had an apparent molecular mass of approx 8 kDa. It was identified as MTs-containing peak basing upon its spectral features, thermostability and molecular weight [6, 16] (Fig. 1*A*). In samples C, ME and N, it was not accompanied by other peaks demonstrating the absence of the conditions for olygomerization or destruction of the MTs. However, the eluent contained both mercaptoethanol and azide (ME+N), and the additional low molecular weight peak was indicated attesting partial breaking of the MTs, probably to separate domains [13].

The D_{254}/D_{280} ratio of the low-weight fraction was in the range 2.7-3.2 in all samples. However, the absorption spectra in the ultraviolet spectral region (UV-spectra) of this fraction were different (Fig. 1*B*). Moreover, in the eluent containing both mercaptoethanol and azide (ME+N), the fraction lost the typical peak intrinsic for the metal-thiolate clusters, while in the sample containing only azide (N), this peak was less manifested than in the control and only mercaptoethanol (ME) contained eluents. Additionally, in control, the maximum of this peak shifted in the middle UV-light region from the typical position. Therefore, according to the chromatographic properties the eluent containing was the most appropriate for elution of the metalated MTs.

Sodium azide (0.02–0.03%) is usually applied to conserve the Sephadex as an inhibitor of the microbial growth. Correspondingly, the utilizing of buffer with its presence cannot be excluded in the repeatedly utilized Sephadex. Current study demonstrated that careful removing of azide from the medium for elution is a decisive condition for the appropriate elution of the MTs.

We detected the metal composition of the MTs-containing peak (Tabl. 1, 2, Fig. 2).

The results of analysis of metal accumulation in tissues of the mollusk from the field groups have shown the inter-site differences (Tabl. 1): the concentration of Cu was the highest in the groups from the small HPP. The concentration of Cd was distinctly lower in the group, sampled downstream the dam of small HPP, than in all other groups. Metal concentration in the MTs-containing chromatographic peak and its ratio to the total concentration of the corresponding metal in the tissue was also site-dependent.

In the depurated mollusks, in each case of the elution Zn was the main metal in the composition, and in the sample N, its part in the ratio was the highest. In the presence of ME, the level of Zn and Cu in the MTs was the lesser. The ratio of Cu and Cd was different due to variations in the Cu content in the peak, whereas the level of Cd was stable. This variability can be explained by the reducing effect of the ME and support of true metal-thiolate cluster structure. In the absence of ME, the non-specific bounding of Zn and Cu in the peak can be expected (so called hypermetalated state) [18, 14]. On the other hand, binding of Cd as the most tightly bound to MTs ions was not dependent on the conditions of the elution.

Comparison of the metal ratio in the MTs and total metal in the tissue indicate a great level of the MTs in the metal binding. Importantly, the mollusks from the less polluted site associated with the micro HPP were able of binding higher part of metals in the MTs than the specimens from the polluted area of the small HPP in a low portion of the river. This phenomenon was described earlier for accumulation of Cd in the freshwater mollusks [15, 16]. It can be explained by the best ability for the metal transport and protein synthesis in the healthy organism. The comparison of metals in the composition

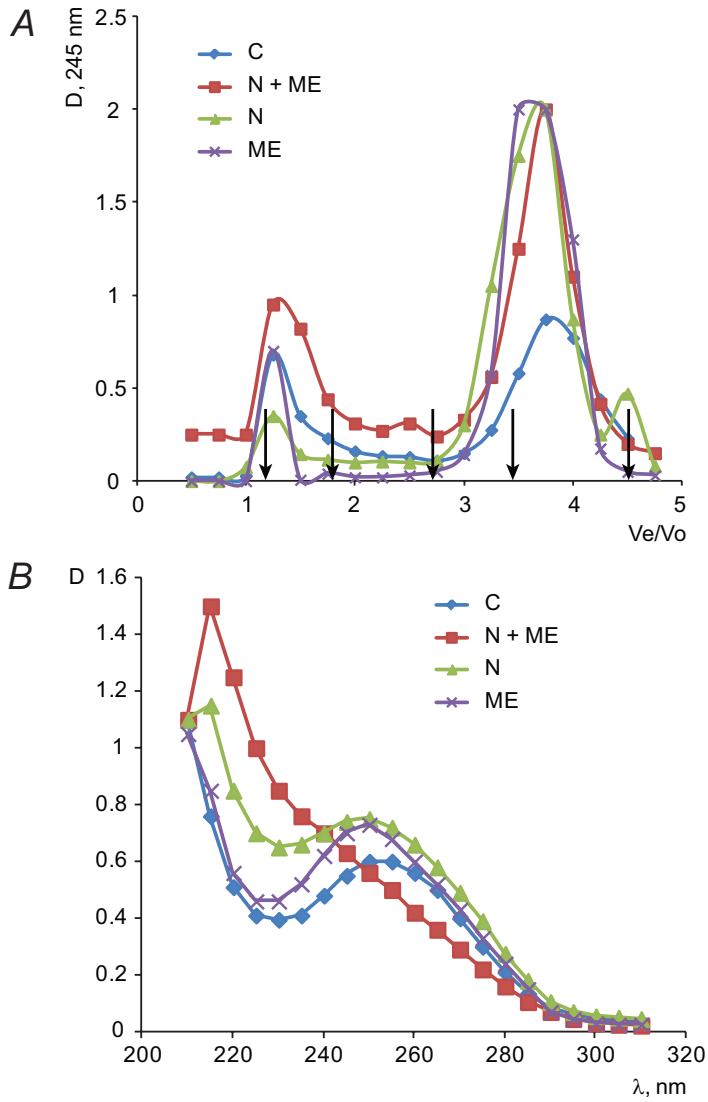

Fig. 1. Chromatographic features of a thermostable extract of the digestive gland of *U. tumidus* from the reservoir of Kasperivtsy HPP after 21 days of the depuration of mollusks: the elution profiles on Sephadex G-50 (*A*) and UV-spectra of low molecular weight fraction (*B*)
Comment. Arrows highlight the elution volume of the molecular weight markers: 25.8 kDa, 17.0 kDa, 12.3 kDa, 8.4 kDa, 3.4 kDa appropriate to 1.1; 1.8; 2.75; 3.3; 4.5 Ve/Vo, correspondingly; Ve, elution volume; Vo, void volume of the column. Samples: without additions (C); containing 10 mM 2-mercaptoethanol and 5 mM sodium azide (ME+N); containing 5 mM sodium azide (N); containing 10 mM 2-mercaptoethanol (ME)

Рис. 1. Хроматографічні риси термостабільного екстракту з травної залози *U. tumidus* із резервуару Касперівської ГЕС після 21 доби депурації молюсків: профіль елюції на Сефадексі G-50 (*A*) та УФ-спектри низькомолекулярної фракції (*B*)
Примітка. Стрілки позначають об'єм елюції маркерів: 25,8 кДа, 17,0 кДа, 12,3 кДа, 8,4 кДа, 3,4 кДа мають параметри елюції 1,1; 1,8; 2,75; 3,3; 4,5 Ve/Vo відповідно. Зразки: без доданків (C); з 10 мМ 2-меркаптоетанолом та 5 мМ азидом натрію (ME+N); з 5 мМ азидом натрію (N); з 10 мМ 2-меркаптоетанолом (ME)

of MTs from the field mollusks and the specimens applied to depuration indicated the efflux of Cu from the MTs, whereas the level of Cd was increased. It could be explained by a unique ability of the MTs to bind almost all Cd in the tissue, whereas the Cu is easy transported from the MTs to other cellular targets [1–3, 9, 20].

Table 1. **Results of metal accumulation in tissue of a digestive gland and its metallothioneins in *U. tumidus* from four field sites, μg×g^{-1} FW (% of total), M±SD, n = 6 for total metal in the tissue and n = 3 for the metallothioneins**

Таблиця 1. **Акумуляція металів у тканині травної залози та її металотіонеїнах у *U. tumidus* із чотирьох місцевостей, мкг×г$^{-1}$ вологої маси тканини (% від загального), M±SD, n = 6 для загального вмісту металу у тканині та n = 3 для металотіонеїнів**

Metal	Groups			
	KU	KD	ZHU	ZHD
Zn total	91.2±1.2^{a}	85.5±1.4^{a}	62.9±1.3^{b}	98.2±0.96^{a}
Zn MTs	37.1±2.9^{a} (40.7%)	20.06±1.5^{b} (23.5%)	28.8±2.3^{c} (45.8%)	27.47±1.4^{c} (28.0%)
Cu total	3.39±0.37^{a}	3.12±0.32^{a}	1.37±0.34^{b}	2.12±0.69^{c}
Cu MTs	1.27±0.15^{a} (58.1%)	0.80±0.09^{b} (25.6%)	1.17±0.2^{a} (85.4%)	1.79±0.22^{c} (84.4%)
Cd total	1.25±0.34^{a}	0.29±0.06^{b}	0.61±0.16^{c}	1.06±0.14^{a}
Cd MTs	0.24±0.02^{a} (19.2%)	0.25±0.023^{a} (86.2%)	0.36±0.04^{b} (59.0%)	0.40±0.03^{b} (37.7%)

Comment. For each parameter, same letters correspond to values that are not different significantly, always P>0.05. Groups: Kasperivtsi small HPP upper and downstream dam groups (KU and KD groups correspondingly), Krasnostavtsi micro HPP upper and downstream dam groups (ZhU and ZhD groups correspondingly)

Примітка. Для кожного параметра однакові букви відповідають значенням, які не відрізняються вірогідно між собою, завжди P>0,05. Групи: Касперівська мала ГЕС вище та нижче дамби (KU та KD групи відповідно), Красноставська мікро ГЕС вище та нижче дамби ((ZhU та ZhD групи відповідно)

Table 2. **Metal composition of the metallothioneins in a digestive gland of *U. tumidus* depending on the eluent, μg×g^{-1} FW, M±SD, n = 3**

Таблиця 2. **Склад металів у металотіонеїнах травної залози *U. tumidus* залежно від елюента, мкг×г$^{-1}$ вологої маси тканини, M±SD, n = 3**

Metal	Samples			
	C	ME+N	N	ME
Zn	42.4±2.8^{a}	20.9±2.8^{b}	86.3±3.7^{c}	35.7±1.9^{d}
Cu	0.75±0.12^{a}	0.21±0.05^{b}	0.21±0.04^{b}	0.11±0.03^{c}
Cd	0.71±0.12^{a}	0.71±0.14^{a}	0.84±0.12^{a}	0.69±0.11^{a}

Comment. Samples: without additions (C); containing 10 mM 2-mercaptoethanol and 5mM sodium azide (ME+N); containing 5 mM sodium azide (N); containing 10 mM 2-mercaptoethanol (ME)

Примітка. Зразки: без доданків (C); з 10 мМ 2-меркаптоетанолом і 5 мМ азидом натрію (ME+N); з 5 мМ азидом натрію (N); з 10 мМ 2-меркаптоетанолом (ME)

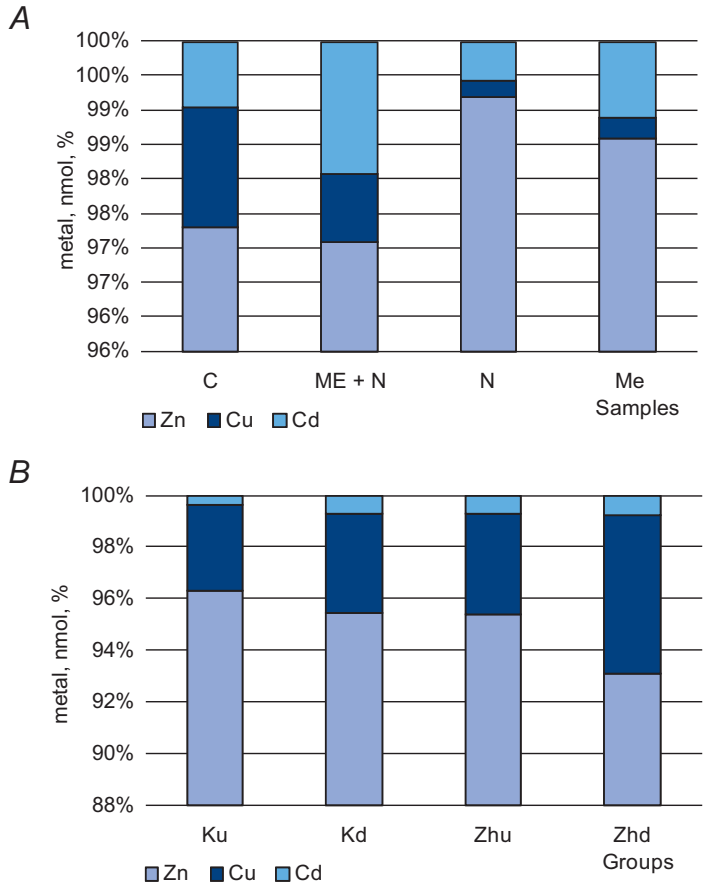

Fig. 2. Metal composition of the metallothioneins in a digestive gland of *U. tumidus* in different eluents (*A*) and depending on the origin of population (*B*), Zn:Cu:Cd, atoms rate (%).
Comment. Samples: without additions (C); containing 10 mM 2-mercaptoethanol and 5 mM sodium azide (ME+N); containing 5 mM sodium azide (N); containing 10 mM 2-mercaptoethanol (ME)

Рис. 2. Склад металів у металотіонеїнах травної залози *U. tumidus* у різних елюентах (*A*) та залежно від походження популяції (*B*), атомні частки Zn:Cu:Cd (%).
Примітка. Зразки: без доданків (C); з 10 мМ 2-меркаптоетанолом та 5 мМ азидом натрію (ME+N); з 5 мМ азидом натрію (N); з 10 мМ 2-меркаптоетанолом (ME)

At least four important conditions to ensure a stability of MT extracted from tissue samples during storage were selected. Heat-treatment of homogenates, storage of supernatants at -70 °C, the addition of a thio-protecting agent to the supernatants, and maintenance of sample dilution to below 1:5 of supernatants were used [5]. In present study, the importance of the maintaining of the composition of the eluent (the avoiding of sodium azide) was stressed. Our results have shown that the composition of metals in the MTs can be substantially changed by the depuration of animals, and Cu is a substance that can easily be removed from these proteins during withstanding of the specimens in the laboratory conditions. One can suggest a necessity of the azide removing from the eluent and keeping the presence of mercaptoethanol for supporting of the MTs metal-binding state.

This work has been granted by the Ministry of Education and Science of Ukraine (Projects 132B and M/35 for O. Stoliar as a Principal Investigation).

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ВМІСТ МЕТАЛІВ У МЕТАЛОТІОНЕЇНАХ ДВОСТУЛКОВОГО МОЛЮСКА *UNIO TUMIDUS* ЗА РІЗНИХ УМОВ ЕКСПОЗИЦІЇ *IN SITU* ТА *IN VITRO*

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Металотіонеїни – це низькомолекулярні протеїни, залучені до гомеостазу і детоксикації перехідних металів. Двостулкові молюски відомі як найефективніші організми-фільтратори, що забезпечують акумуляцію токсичних металів із водного середовища. Аналітичні методи виявлення та кількісного визначення металотіонеїнів у біологічних зразках потребують вдосконалення для досягнення коректних умов визначення складу їхніх металів. Азид натрію часто використовують для консервації Сефадексу та запобігання росту мікрофлори. Мета цієї роботи – обрати умови для виділення металотіонеїнів двостулкового молюска, які забезпечать стабільність їхніх метал-депонувальних форм. Було досліджено металотіонеїни молюсків із чотирьох місцевостей у басейні ріки Дністер – вище та нижче дамб двох гідроелектростанцій у басейні р. Дністер (малої ГЕС у Касперівцях на р. Серет і мікроГЕС у Красноставцях на р. Жванчик). Молюсків із найбільш забрудненої місцевості (резервуар малої ГЕС) піддавали депурації в лабораторії протягом 21 доби, після чого металотіонеїни травної залози виділяли як термостабільні протеїни та порівнювали за різних режимів елюції. Було застосовано чотири склади елюенту на основі 10 мМ Трис-HCl буферу, pH 8,0: без добавок, з додаванням 10 мМ 2-меркаптоетанолу та 5 мМ натрій азиду; з додаванням лише 5 мМ натрій азиду або 10 мМ 2-меркаптоетанолу. Встановлено, що наявність азиду в елюенті призводить до

спотворення хроматографічних і спектральних характеристик металотіонеїнів. Визначення складу металів у металотіонеїнах встановило, що вони зв'язують переважно цинк. Із досліджених груп молюсків із ділянок ГЕС найвищий вміст купруму у тканині травної залози та металотіонеїнах був відзначений у молюсків із резервуару малої ГЕС. Проте депурація молюсків викликала елімінацію купруму з металотіонеїнів. Концентрація цинку та кадмію була досить стабільною у всіх досліджених умовах елюції. Детоксикаційна функція металотіонеїнів щодо кадмію та купруму найгірше виражена у молюсків із резервуару малої ГЕС. Отримані результати привертають увагу до необхідності дотримання коректних умов елюції у дослідженні металотіонеїнів молюсків.

Ключові слова: металотіонеїни, двостулкові молюски, купрум, цинк, кадмій, гель-розподільча хроматографія

Одержано: 12.03.2019