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M. NEMERKO, K. YUNKO, O. STOLIAR

[1]Ternopil Volodymyr Hnatiuk National Pedagogical University
M. Kryvonosa Str., 2, Ternopil, 46027
E-mail: Oksana.Stolyar@tnpu.edu.ua

THE EFFECT OF ZINC AND RARE EARTH ELEMENTS ON METALLOTHIONEIN OF BIVALVE MOLLUSC RECONSTITUTED *IN VITRO*

Rare earth elements (REEs) are increasingly attracting attention as new pollutants due to their growing use in various industries, leading to environmental contamination. Metallothioneins (MT) are low molecular weight proteins rich in cysteine that are capable of binding d-metals, both essential and toxic. Since the REEs exhibit the ionic mimicry with the essential metals, their ability to bind to MT can be expected. However, data on the utilizing of MT as a molecular targets of REEs, in particular gadolinium (Gd^{3+}) and yttrium (Y^{3+}) are currently lacking. Bivalve molluscs belong to the main filtered organisms in the freshwater bodies, and, consequently, they can accumulate the REEs in their tissues. Therefore, the aim of this study was to compare the ability of MT to reconstruct their native structure with the essential metal Zn^{2+} and representative REEs Gd^{3+} and Y^{3+} utilizing the UV-spectral peculiarities of MT. To do this, MT was isolated from the digestive gland of bivalve mollusc *Unio tumidus* using gel permeable chromatography and subjected to demetalation by 0.05 M HCl. Resulted partially metallated metallothionein (pMT) was subjected to chromatography again and incubated with Zn^{2+}, Gd^{3+} and Y^{3+} ions for 60 min with the aim to reconstitute the metallothionein during 1 h (MTr 1 h) and 24 h (MTr 24 h). MTr was exposed to H_2O_2 in two concentrations (0.5 mM and 5 mM) for 1 h to assess the stability of thiolate clusters. For all utilized forms (MT, pMT, MTr 1h, MTr 24 h) UV-spectra were determined and their indexes of absorption were calculated.

The results of the study indicated partial demetallisation of MT in an acidic solution. Incubation with each of the three metals for 1 hour led to a decrease in spectral intensity at 255 nm, with Gd-MTr causing the greatest decrease in spectral intensity in this range. The decrease at 215–220 nm (in the blue shift region) indicated the ability of these metals to influence zinc binding. All types of MTr exhibited virtually identical spectral absorptions across all ranges after 24 hours. Furthermore, over 24 hours, Zn-MTr significantly altered the absorption of MT, which may indicate a redistribution of metals, whereas the absorption for Gd and Y MTr was not changed prominently.

Following treatment with 0.5 mM H_2O_2 for 1 hour, Gd-MTr showed an increase of absorption at 255 nm, whilst Zn-MTr showed a strong increase in absorption at 215–220 nm. Y caused a decrease in this area of spectra. Exposure to 5 mM H_2O_2 led to an increase in absorption at 255 nm in Zn-MTr and Gd-MTr. Particularly, Y-MTr exhibited the greatest decrease in spectral absorption in these ranges, indicating the susceptibility of Y-MTr to oxidation, whilst Gd-MTr exhibited the highest spectral peak at 255 nm.

Summarising, basing on the spectral features, we confirmed the ability of Gd and Y to reconstruct the metal-thiolate clusters similar to Zn-thiolate clusters, typical for Zn-MT. The reconstituted Gd-MTr were more stable for the oxidation than Y-MTr.

Keywords: Metallothionein, apothionein, gadolinium, yttrium, UV-spectrum.

Rare earth elements (REEs) are widely used in various industries due to their special chemical and physical properties. Their use leads to serious environmental pollution. Many studies show the negative impact of REEs, in particular Gd^{3+} and Y^{3+}, on the redox state of aquatic animals [1, 6]. However, the molecular targets of their binding within the cells are not studied thoroughly [2].

Metallothioneins (MT) are thermostable low molecular weight proteins (6-8 kDa). These proteins have a unique structure and properties completely lacking aromatic amino acids (tyrosine, tryptophan, phenylalanine). On the other hand, they are rich in cysteine (~30%) providing a high ability to bind metals, forming in the typical MT two metal-thiolate clusters [17]. Correspondingly, they have particular spectral features, lacking the typical for most proteins 280 nm absorption peak and exhibits a characteristic shoulder at 250 nm, which together with the absence of any significant absorbance at 280 nm, has been used as an indicator of the MT presence. However, the kind of bounded metal influences the features of typical peak. Therefore, the particular metal composition of MT can be identified by their UV-spectrum characteristics [3, 18].

Since the MT are involved in the binding and transport of irreplaceable physiological metals zinc (Zn^{2+}) and copper (Cu^{+}) and the detoxification of toxic metals cadmium (Cd^{2+}) and mercury (Hg^{2+}) regulating their homeostasis [14], the analysis of UV-spectra of MT are crucially important for the identification of their metal composition. Besides, MT are also involved in many physiological and pathological processes, due to the ability to absorb reactive oxygen species (ROS), and they are highly sensitive to the presence of oxidants in the medium [4, 16].

Zn^{2+} is one of the most important essential metals in living organisms and the most common metal associated with MT. Zn^{2+} is also a common cofactor that enables the functioning of many enzymes [8]. A deficiency or excess of free Zn^{2+} can lead to many pathologies, so it is important to regulate its concentration within the cell. Particularly, bound Zn^{2+} in MT plays an important role as an indicator of redox status. For example, during oxidative stress, ROS can release Zn^{2+} from MT, which in turn leads to an increase in its concentration within the cell, activating a transcription factor (MTF-1) that has domains for Zn^{2+} binding. The binding of Zn^{2+} to MTF-1 leads to its activation and an increase in the total amount of MT capable of absorbing ROS. Heavy metals such as Cd^{2+} and Hg^{2+} are capable of displacing Zn^{2+} from MT, which leads to an increase in MT synthesis and heavy metal binding [7, 10, 12].

Since the REEs exhibit the ionic mimicry with the essential metals [2], their ability to bind to MT can be expected. However, data on the utilizing of MT as a molecular targets of REEs, in particular gadolinium (Gd^{3+}) and yttrium (Y^{3+}), are currently lacking. It is therefore important to elucidate whether the MT can bind these metals. Bivalve molluscs belong to the main filtered organisms in the freshwater bodies, and, consequently, they can accumulate the REEs in their tissues [1]. Therefore, the aim of this study was to compare the ability of MT to reconstruct their native structure with the essential metal Zn^{2+} and representative REEs Gd^{3+} and Y^{3+} utilizing the UV-spectral peculiarities of MT.

Materials and methods

Sample preparation and obtaining a thermostable fraction. Metallothioneins were isolated from the digestive gland of bivalve molluscs *Unio tumidus* (Philipson, 1788) using gel permeation chromatography of the thermostable fraction of proteins. To obtain a homogenate, tissue samples from five animals were combined and homogenised (1:10 w/v) in 10 mM Tris-HCl buffer (pH 8.0) containing 10 mM 2-mercaptoethanol (Sigma-Aldrich) to prevent oxidation of SH-groups and 0.1 mM inhibitor phenylmethylsulfonyl fluoride protease (Sigma-Aldrich) as it was described in Falfushynska et al. (2010) [3]. The homogenate was centrifuged for 45 minutes at 16,000 g and 4ºC. The resulting supernatant was exposed to a temperature of 85°C for 5 minutes to precipitate thermolabile proteins, after which centrifugation was repeated under similar conditions. The resulting supernatant containing thermostable proteins was fractionated by gel filtration on a chromatography column (50×1.5 cm) packed with Sephadex G-50 (Sigma-Aldrich). Elution was performed by Tris-HCl buffer (pH 8.0) with the addition of 10 mM 2-mercaptoethanol at a rate of 0.33 ml/min. The eluent did not contain EDTA to prevent the loss of metals bound to proteins. Twenty fractions of 5 ml each were collected.

Identification of MT. The optical density of the fractions was measured using a ULAB 102 spectrophotometer at wavelengths of 254 nm and 280 nm. The chromatographic column was calibrated using proteins of known molecular weight: albumin (67 kDa), chymotrypsin (25 kDa), cytochrome C (12.3 kDa), insulin (5.8 kDa) (Sigma-Aldrich markers were used). MTs were identified as thermostable low-weight proteins characterised by a high D254/D280 ratio. The two fractions with the highest MT content were combined (total volume 10 ml) and their UV spectrum was recorded.

Obtaining demetalated thioneins and their reconstruction. To obtain the apo form of MT (apo-MT), the isolated fraction containing MT was repeatedly eluted on a Sephadex G-50 column (50×1.5 cm) using a 0.05 M HCl solution (elution rate 0.5 ml/min, fraction volume 5 ml). Detection was performed at 220 nm [3]. The fraction containing apothionein was used to reconstruct metallothionein by incubation with a freshly prepared 0.6% solution (132 mg of $ZnSO_4 \cdot 7H_2O$, 71 mg of $GdCl_3 \cdot 6H_2O$ and 102 mg of $YCl_3 \cdot 6H_2O$) were added. of rare earth elements (Gd, Y) and Zn salts in 0.1 M deaerated HCl containing 4% NaCl. The incubation lasted 60 minutes, after which the UV spectra of the obtained fractions were recorded.

Determination of oxidative modifications of MT. Oxidation of MT *in vitro* was modelled by incubating the purified MT fraction with H_2O_2 solutions (0.5 mM and 5 mM) at 25 °C for 60 minutes, after which the UV spectrum was recorded. For all utilized forms of MTs, the indexes of absorption D215/D230, D260/D230, D245/D280, D255/D280, D245/D295 were calculated to indicate the changes in the typical spectra characteristics.

Results and discussion

MT isolation. Using gel permeable chromatography, two fractions were isolated from the supernatant of mollusc digestive glands: a high molecular weight fraction (~67 kDa), which may indicate incomplete isolation of high molecular weight compounds, and a low molecular weight fraction (~6 kDa). The low molecular weight fraction had all the characteristics of MT, namely: molecular weight of the fraction about 7 kDa, thermostability (no denaturation occurred at 85-90°C for 5-10 min), specific UV absorption of about 254-255 nm, which is characteristic of metal-thiolate clusters, and no absorption at 280 nm, indicating the absence of aromatic amino acids in the protein composition [5].

Apothionein is a form of MT that is unbound to metals and is characterised by a lack of absorption above 230 nm. This has made it possible to use UV absorption above 230 nm to detect the content of certain metals in MT. For example, Cd-MT has a specific spectral absorption at 250 nm, which allows its presence in MT to be determined. The shoulder in the UV spectrum below 240 nm is characteristic of Zn-MT [15].

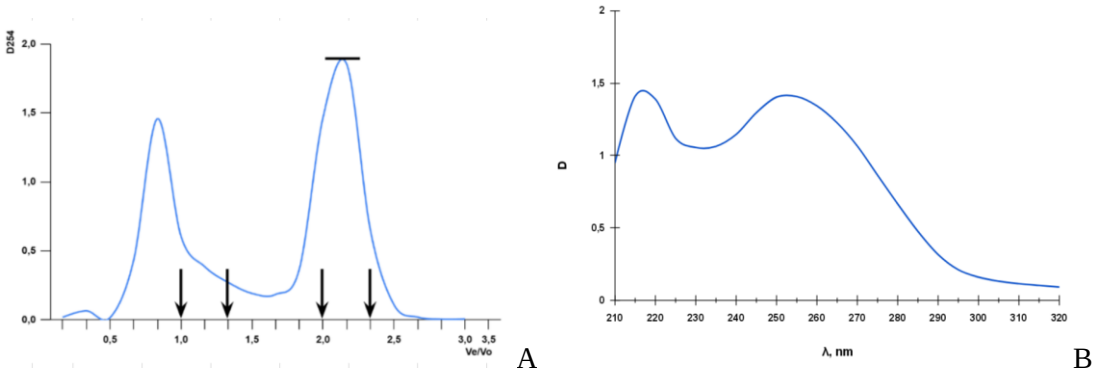


Fig. 1. Elution profiles (A) and UV-spectra (B) of thermostable proteins from the digestive gland of a mollusc.

Note: in Fig. 1 A, the arrows indicate the elution volume of markers: albumin (66 kDa) 1.0; chymotrypsin (25 kDa) 1.33; cytochrome c (12 kDa) 2.0; insulin (5.8 kDa) 2.33. Ve – elution volume; Vo – external gel volume.

Isolation of pMT. After isolation, MT was subjected to gel permeable chromatography, after which one fraction with a molecular weight of approximately 6 kDa was isolated.

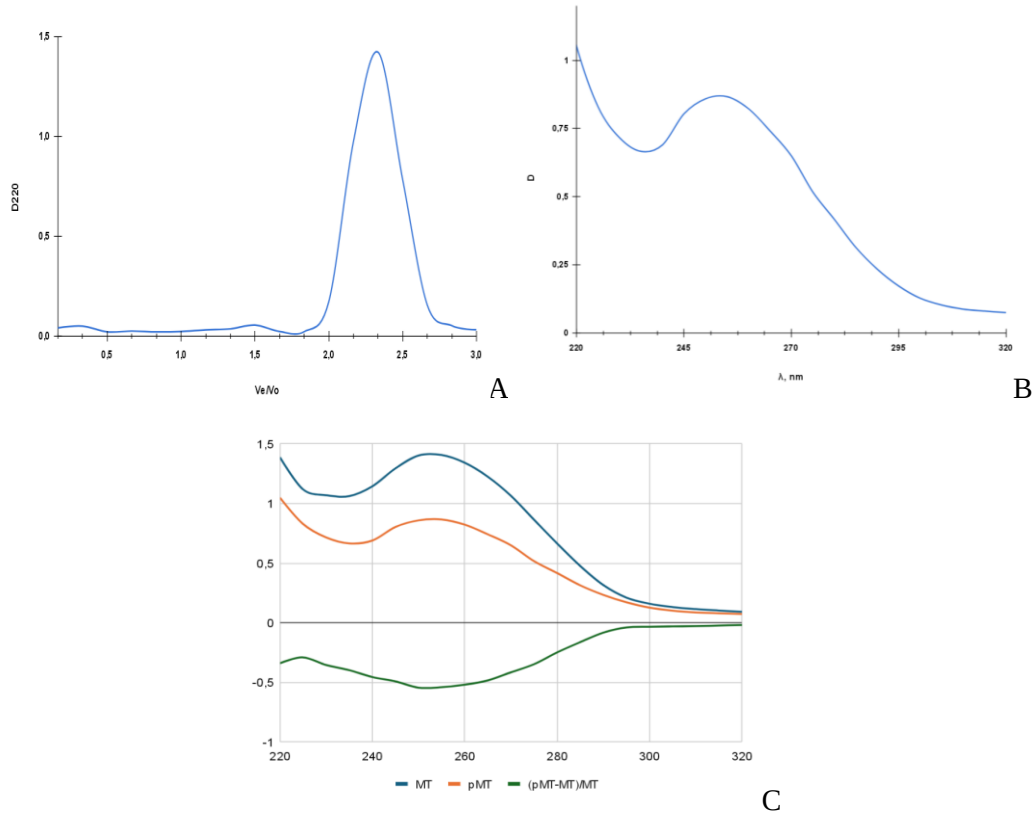


Fig. 2. Elution profiles (A), UV spectra (B), and difference between the spectra of metalationeins and purified thionein (C) in the digestive gland of a mollusc.

However, as can be seen in Fig. 2 B, the peak at 220 nm disappeared and the peak at 255 nm decreased. This indicates partial removal of metals from MT without the formation of the apo-MT, indicating a strong stability of MT to the effect of 0.05 M HCl.

Incubation of pMT with Zn, Gd and Y. After receiving, pMT was incubated in the presence of Zn^{2+}, Gd^{3+} or Y^{3+} for 1 h and 24 h, respectively. During the exposure to each of three metals for 1 h, the spectral absorption was analysed.

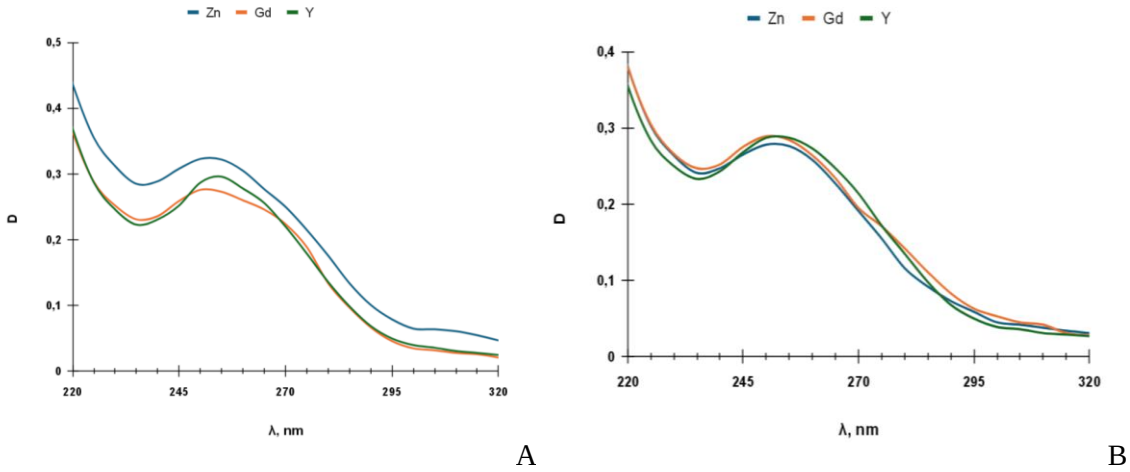


Fig. 3. UV spectra of apo-MT after incubation with Zn, Gd, and Y for 1 h(A) and 24 h (B).

It is important to note that the secondary structure of MT depends directly on the metals to which it is bound [13, 15]. The UV-spectra indicates the correspondent particularities. Particularly, the

elevated absorption of UV radiation at a wavelength of 250 nm, typical for Cd-MT, occurs during charge transfer from the ligand to the metal (LMCT). The absorption of UV radiation at a wavelength of 255 nm characterises Cu-MT. This difference is explained by the ability of Zn-MT and Cd-MT to form tetrahedral structures, whereas Cu-MT form various structures, such as tetrahedral, trigonal and diagonal geometries, where different geometric structures can influence the absorption spectra. Interestingly, after adding Zn^{2+} to pMT, there was no increase in the characteristic absorption at 215-240 nm, on the contrary, absorption in this range decreased. It is worth of mention, that the use of UV spectra to detect metals in heteronuclear clusters is problematic because the identity of metal chromophores is often disrupted, and it is difficult to separate the contribution of each metal in the spectra [15].

Consequently, it is currently very difficult to draw definitive conclusions about the structure of the formed clusters and to explain the exact nature of the UV spectra at 220 nm and 255 nm, because, according to the spectral peculiarities, the MT were only partially demetalated under the step of depuration. This may be due to different *in vitro* Zn/M ratios and bond ratios (Zn-S-M/Zn-S-Zn), as well as the type of clusters formed [13]. However, the addition of Zn^{2+} to MT causes noticeable changes in the spectra that are characteristic of cluster structures, indicating a change or distribution of metal-thiolate clusters. The absorption in the 215 nm range is also due to the presence of peptide bonds, which may indicate a change in the nature of the peptide due to the interaction of MT with metals [15].

Since clusters in MT are thermodynamically stable and kinetically labile, this allows intramolecular exchange between metals and their redistribution. A change in absorption at 220 nm was noticeable 24 h after incubation, where Zn^{2+} showed the greatest change in this spectrum compared to Gd^{3+} and Y^{3+}, which may indicate a redistribution of metals characteristic of heteronuclear clusters (Zn,Cd/Cu-MT) [9]. But the redistribution of the metal may also depend on the conditions (*in vitro* or *in vivo*). In contrast, for Gd^{3+} and Y^{3+} such strong changes in the spectra were not observed.

The affinity of metal ions for MT depends on many factors. One of the main factors is the concept of hard and soft acids and bases (HBAS). It is known that soft metal ions prefer to bind with soft bases, one of which is sulphur. Most metals, such as Zn^{2+}, $Cd,^{2+}$ Cu^{+} and Hg^{2+}, are considered soft or semi-soft acids, whilst Gd^{3+} and Y^{3+} are hard acids. Therefore, they have a high affinity for binding to strong bases, such as carboxylates (O) and amines (N), meaning they are oxyphilic. One study has shown that lanthanum, an element from the REE group, is capable of binding to MT via carboxyl groups, as well as inducing MT expression [19]. This suggests that MT has the ability to bind even to metals that are atypical for it, through other amino acid residues (aspartic and glutamic acids), which likely affects the stability of the primary cluster structures in MT. In addition, the stability of metal-thiolate clusters in MT is influenced by the binding enthalpy, entropy, and the biomolecular environment itself [9].

The factors mentioned above clearly indicate that Gd^{3+} and Y^{3+} are not typical metals that usually bind to MT, particularly via cysteine residues, and are more likely to prefer binding to carboxyl groups in MT. However, as shown in Fig. 2 A, their presence altered the spectra characteristic of MT cluster structures. Within 1 h, their spectra changed, whilst the spectra at 255 nm were almost identical for all groups over a 24 hour period.

The effect of H_2O_2 on Zn/Gd/Y-MTr. It is known that during oxidative stress, ROS can affect SH-groups, promoting the oxidation of M-S sulphur, which leads to the release of metals from MT. Each metal has a different affinity for MT under oxidative conditions, for example, Zn^{2+} has a lower affinity for MT compared to Cd^{2+} and Cu^{+}. As mentioned earlier, ROS and Cd^{2+} can displace Zn^{2+} from MT, promoting an increase in the concentration of cellular Zn^{2+}, which can bind to the transcription factor (TFM-1) and activate MT synthesis, promoting an increase in total MT to protect against ROS and/or Cd^{2+} by binding these agents [7, 12].

After the addition of 0.5 mM and 5 mM H_2O_2, completely different spectra are observed for Zn/Gd/Y-MTr. For Gd-MTr, following the addition of 0.5 mM H_2O_2, the spectral absorption intensity in the 215–220 nm range remained virtually unchanged, whereas at 255 nm it increased. The spectra of Gd-MTr also show noticeable changes in the 230–240 nm range, which can be explained by a certain effect of H_2O_2 on the MT structure. Following exposure of Gd-MTr to 5 mM H_2O_2, an increase

in the intensity of spectral peaks in the 215–220 nm and 255 nm ranges was observed. For Zn-MTr, a marked increase in spectral absorption at 215–220 nm and 255 nm was observed following the addition of 0.5 mM. After addition of 5 mM H_2O_2 to Zn-MTr, an increase in peak intensity at 255 nm was observed, while the spectra at 215–220 nm remained virtually unchanged. In Zn-MTr, the peak at 255 nm was smaller, whereas in Gd-MTr it was the most pronounced among all groups upon the addition of 5 mM H_2O_2. The situation for Y-MTr is quite the opposite: after the addition of 0.5 mM H_2O_2, its spectral absorption in the 215–220 nm and 255 nm ranges decreased slightly.

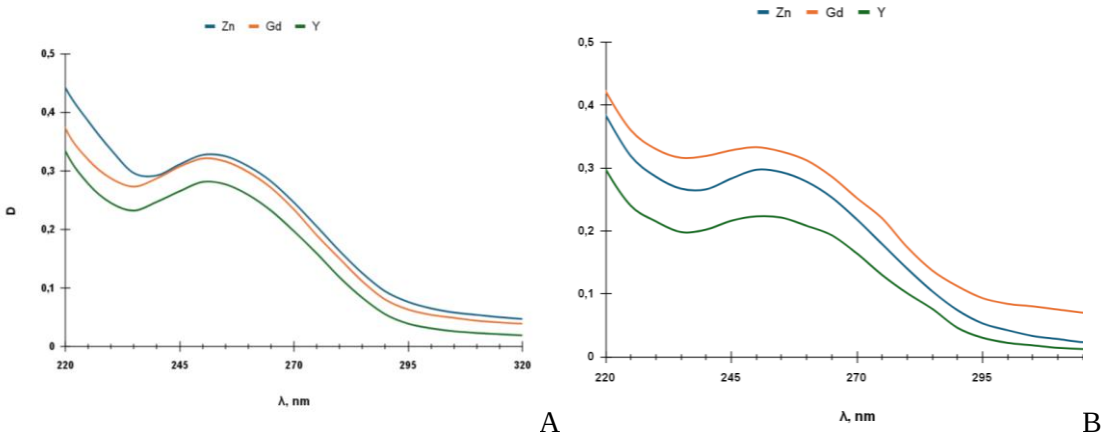
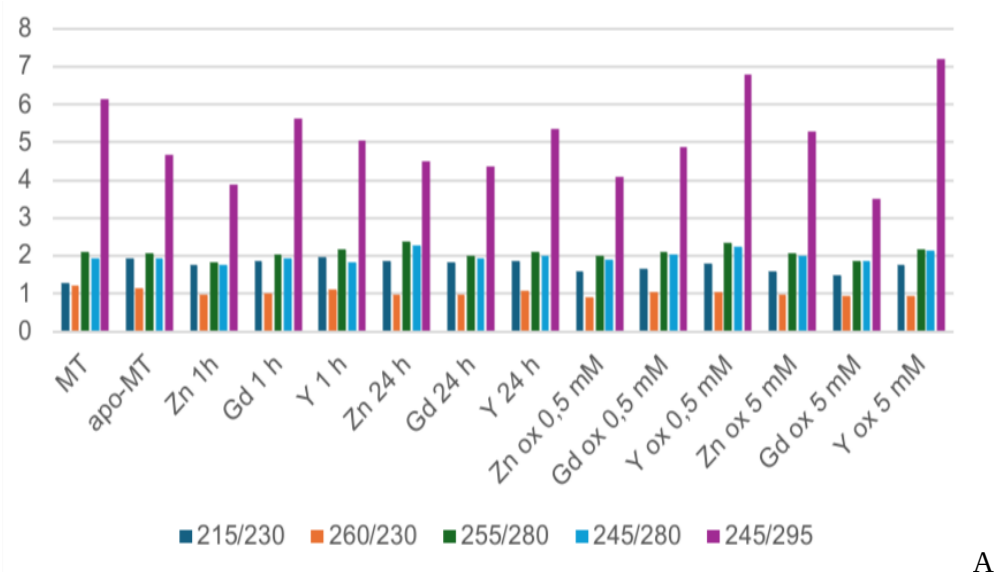
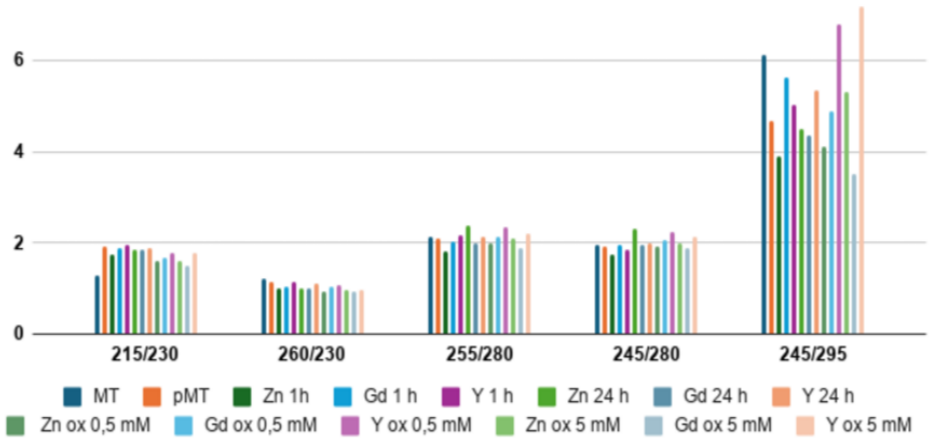


Fig. 4. UV spectra of MT after incubation of H_2O_2 with Zn/Gd/Y-MTr for 1 h at concentrations of 0.5 mM (A) and 5 mM (B).

Under the influence of 5 mM H_2O_2, a decrease in the intensity of the typical UV-peak was observed in Y-MTr. Probably, Y, together with H_2O_2, affected the stability of the MT cluster structure and made it more sensitive. These results indicate that although Gd^{+3} and Y^{3+} have similar chemical properties, their effect on the stability of MT cluster structures is very different. The different changes in the spectra at 220 nm and 255 nm following exposure to H_2O_2 may indicate the ability of these metals to influence MT, as these spectra are predominantly determined by the presence of M–S bonds.





B

Fig. 5. Ratios of UV absorption of all studied forms of MT: A – for each form separately; B – for each index separately.

The calculation of the ratios of UV-absorption indicated clearly the differences between the spectra of all forms of MT (Fig. 5). In all reconstructed forms the blue shift was detected, attesting the increased role of Zn among the metals in protein. The indexes 260/230 and 245/280 changed in a low limits, but most remarkable in the ZnMTr ox (260/230). The rate of 255/280 indicated the loss on typical for cluster peak with the maximum at 255 nm. However, in the partially demetalated form this peak was shown. Hence, the total demetalation of MT was not achieved. Most remarkable differences were shown for D245/D295 ratio highest values for MTr of Gd and Y, and particularly for YMTr ox that witness the substantial impact of the studied REEs on the MT stability.

Conclusion

The results of the study showed the ability of Gd^{3+} and Y^{3+} to change the structure of MT. As mentioned above, the secondary structure of MT depends on its binding with metal, therefore, based on the data obtained, where the influence of all three metals caused changes in the spectra responsible for the structure and cluster nature of MT, it can be concluded that Gd^{3+} and Y^{3+} are capable of influencing the cluster structure of MT. Furthermore, upon incubation of Zn/Gd/Y-MTr with H_2O_2, each group exhibited a different reaction.

Importantly, this study did not use plural methods for additional confirmation of S-M connections, but only compared the changes in spectra after the influence of metals on partially metalated MT, which, incidentally, proved to be very informative. The conditions *in vitro* can also be distinct from the situation *in vivo* with the possibility of REE to bind to MT. Although the spectra after adding Zn^{2+} to the partially metalated MT were atypical and the properties described above cannot fully explain why such spectra were formed, based on the data we obtained, we can assume that Gd^{3+} and Y^{3+} are capable to influence the structure of MT.

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М. Немерко, К. Юнко, О. Столяр
Тернопільський національний педагогічний університет імені Володимира Гнатюка, Україна

ВПЛИВ ЦИНКУ ТА РІДКІСНОЗЕМЕЛЬНИХ ЕЛЕМЕНТІВ НА РЕКОНСТРУКЦІЮ МЕТАЛОТІОНЕЇНУ ДВОСТУЛКОВОГО МОЛЮСКА *IN VITRO*

Рідкісноземельні елементи (РЗЕ) привертають дедалі більше уваги як нові забруднювачі навколишнього середовища у зв'язку з розширенням сфер їх використання та зростанням рівня забруднення екосистем. Металотіонеїни (МТ) є низькомолекулярними протеїнами, багатими на цистеїн, які здатні зв'язувати як есенціальні, так і токсичні d-метали. Оскільки РЗЕ характеризуються явищем іонної мімікрії щодо есенціальних металів, можна припустити їхню здатність взаємодіяти з МТ. Водночас дані щодо взаємодії металотіонеїнів із гадолінієм (Gd^{3+}) та ітрієм (Y^{3+}) наразі відсутні. Двостулкові молюски є основними організмами-фільтраторами прісноводних водойм, тому здатні накопичувати РЗЕ у своїх тканинах. Відтак метою роботи було порівняти здатність МТ відновлювати нативну структуру за участю есенціального металу цинку та представників РЗЕ – гадолінію й ітрію – на основі аналізу ультрафіолетових спектральних характеристик МТ.

Металотіонеїн виділяли з травної залози двостулкового молюска *Unio tumidus* методом гель-проникної хроматографії та піддавали деметалізації у 0,05 М HCl. Отриманий частково деметалізований металотіонеїн (рМТ) повторно очищували та інкубували з іонами Zn^{2+}, Gd^{3+} і

Y^{3+} протягом 60 хв для реконституції металотіонеїну через 1 год (MTr 1 h) та 24 год (MTr 24 h). Для оцінки стабільності тіолатних кластерів реконструйовані форми обробляли H_2O_2 у концентраціях 0,5 і 5 мМ протягом 1 год. Для всіх досліджуваних форм визначали УФ-спектри та розраховували спектральні індекси поглинання.

Встановлено, що кислотна обробка спричиняла часткову деметалізацію МТ. Інкубація з кожним із металів протягом 1 год супроводжувалася зниженням інтенсивності поглинання при 255 нм, причому найбільш виражений ефект спостерігався для Gd-MTr. Зменшення поглинання в діапазоні 215–220 нм свідчило про здатність досліджуваних металів впливати на зв'язування цинку. Через 24 год усі варіанти MTr характеризувалися подібними спектральними показниками. Водночас для Zn-MTr відзначено суттєві зміни поглинання, що може свідчити про перерозподіл металів, тоді як для Gd-MTr і Y-MTr такі зміни були менш вираженими.

Після дії 0,5 мМ H_2O_2 для Gd-MTr спостерігалося збільшення поглинання при 255 нм, тоді як для Zn-MTr – у діапазоні 215–220 нм. Для Y-MTr було характерне зниження поглинання в цій області спектра. Після обробки 5 мМ H_2O_2 для Zn-MTr і Gd-MTr також відзначено збільшення поглинання при 255 нм. Водночас Y-MTr характеризувався найбільшим зниженням спектрального поглинання, що свідчить про його вищу чутливість до окиснення. На підставі спектральних характеристик підтверджено здатність гадолінію та ітрію відновлювати метал-тіолатні кластери, подібні до характерних для Zn-MT. Реконструйовані форми металотіонеїну з гадолінієм виявилися більш стійкими до окиснення порівняно з формами, що містили ітрій.

Ключові слова: металотіонеїн, апотіонеїн, гадоліній, ітрій, УФ-спектр.

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