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Short communication

Differential ESI-MS behaviour of highly similar metallothioneins

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ABSTRACT

ESI-MS can only be accepted as a quantification method when using standards with a high resemblance to the analyte(s). Unfortunately, this is usually not applicable to metallothioneins (MTs), a superfamily of singular metal-binding cysteine-rich proteins, present in all living organisms, since the absence of suitable reference material due to the high diversity among metal–MT species precludes their quantification by molecular mass spectrometry. Even thus, it is widely assumed that the intensities of the ESI-MS peaks of similar species are directly correlated with their relative concentration in the sample, and this has been extended to the determination of different MT proteins coexisting in a sample.

Practically all organisms contain several MT isoforms, some of them exhibiting highly similar sequences, with conserved coordinating Cys residues. For the current analysis, we used as a model system the MT isoforms of two terrestrial snails (*Helix pomatia* and *Cornu aspersum*). Hence, distinct samples were prepared by mixing, at different molar ratios, the recombinant HpCuMT and HpCdMT isoforms from *H. pomatia*, or the recombinant CaCuMT, CaCdMT and CaCdCuMT isoforms from *C. aspersum*, and they were analyzed by ESI-MS both at neutral pH (for Zn-loaded MT forms) and at acidic pH (for the corresponding apo-forms). The results here presented reveal that the ESI-MS peak intensity of a single MT species strongly depends on its sensitivity to be ionized, and thus, on the presence or absence of metal ions bound. Furthermore, our data demonstrate that very similar MT isoforms of the same organism with similar pI (ranging from 7.9 to 8.3) can show a clear different sensitivity to ES ionization, something that cannot be readily predicted only by consideration of their amino acid content. In conclusion, even in this optimum case, deductions about quantity features of MT samples drawn from ESI-MS measurements should be carefully considered.

1. Introduction

Soft ionization molecular mass spectrometry, namely ESI-MS and MALDI-TOF MS, cannot be used for the quantification of different molecules present in a sample, because the intensity of the respective peaks that they yield in the MS spectra depends not only on their respective concentrations, but mainly on their behaviour as ionizable particles. However, in certain occasions, the use of suitable standards made possible the quantification of similar species [1–3]. When analyzing metalloproteins, the difficulties found for quantification purposes are more severe than in the case of other proteins, due to modifications of their pI and/or charge properties introduced by the presence of metal ions bound to the peptide chains. A very special, and highly illustrative case can be observed for metallothioneins.

Metallothioneins (MTs) are a singular superfamily of metal-binding, Cys-rich peptides, present in all living organisms [4,5]. In this case, the common absence of reference material and the high diversity among MT sequences, their promiscuity referring to metal – nature and number – load and other features of the metal–MT species makes virtually impossible to quantify the MT complexes present in a preparation by means of molecular MS. Despite this, many authors, including we among them, assume that the ratios between the peak intensities of similar metal–MT species in an ESI-MS spectrum bear a certain relation to their respective abundance in the sample [6–9]. But, can be this assumed for any MT system? How far can these assumptions be extended to any analysis conditions?

In this communication, we present a detailed overview of the response to ESI-MS of five highly similar MT isoforms, belonging to two terrestrial snails: *Helix pomatia* and *Cornu aspersum*. These organisms synthesize several MT isoforms, which show distinct metal specificity (for cadmium—CdMTs- and copper—CuMTs-binding) despite exhibiting highly similar sequences and full conservation of their Cys residues (Table 1) [10,11].

The use of recombinant techniques allows us to obtain the single protein desired in highly pure and concentrated solutions, and complexed with the desired metal ion. Precisely, in this work, the

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Table 1
Sequences of the recombinant metallothionein isoforms of *Helix pomatia* (HpMT: two isoforms, one specific for Cd and the other specific for Cu) and *Cornu aspersum* (CaMT: three isoforms, Cd, Cu and Cd/Cu without clear specificity) used in this work. The initial GS residues are a consequence of the expression system used, based on fusion protein synthesis and ulterior cleavage [13].

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HpCuMT      GS--GRGKNCGGACNSNPCSCGNDCKCGAGCNCDRCSSCHCSNDDCKCGSQCTGSGSCKCGSACGCK
HpCdMT      GSGKGKGEKCTSACRSEPCQCGSKCQCGEGCTCAACKTCNCTSDGCKCGKECTGPDSCKCGSSCSCK

CaCdMT      GSMSGKGKGEKCTAACRNEPCQCGSKCQCGEGCTCAACKTCNCTSDGCKCGKECTGPDSCKCGSSCGCK
CaCdCuMT    GSMSGKGSA--CAGSCNSNPCSCGDDCKCGAGCSCAQCYSCQCNNDTCKCGSQCSTSGSCKCGS-CGCK
CaCuMT      GSMSGRGQN--CGGACNSNPCNCGNDCNCGTGCNCDQCSARHCSNDDCKCGSQCTRSGSCKCGNACGCK
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snail MT species produced by bacteria grown in Zn-enriched media have been considered. Our goal was the analysis of the ESI-TOF MS spectra recorded when mixing, at different molar ratios, the Zn-complexes of the several MTs of a same snail species, this is, HpCuMT and HpCdMT isoforms from *H. pomatia*, and CaCuMT, CaCdMT and CaCdCuMT isoforms from *C. aspersum*, both at neutral pH (for the analysis of the Zn-loaded MT form peaks) and at acidic pH (for the consideration of the corresponding apo-form signals).

2. Materials and methods

The Zn-complexes of HpCuMT, HpCdMT, CaCuMT, CaCdCuMT and CaCdMT were obtained following the recombinant methodology routinely used by this group [13], and already extensively applied to a considerable amount of MTs [14]. The full characterization of their metal-binding features will be the object of further reports (in preparation), so that this work only refers to the ESI-MS behaviour of their respective Zn-complexes.

The recombinantly expressed MT complexes were analyzed for element composition (S, Zn, Cd and Cu) by inductively coupled plasma atomic emission spectroscopy (ICP-AES) on a Polyscan 61E spectrometer (Thermo Jarrell Ash Corporation, Franklin, MA, USA) at appropriate wavelengths (S, 182.040 nm; Zn, 213.856 nm; Cd, 228.802 nm; Cu, 324.803 nm). Samples were prepared either at "conventional" (dilution with 2% HNO_3 (v/v)) [15], or at "acidic" (incubation in 1 M HCl at 65 °C for 5 min) conditions [16]. MT concentration in the recombinant preparations was calculated assuming that the only contribution to their S content was that made by the MT peptides. In all cases, the RSD in the concentration of S or Zn was lower than 5%, and the amounts of Cu and Cd were always lower than the detection limit.

Several experiments were run with samples at 3 different concentrations: 10, 50 and 100 μM of each protein, which covers the common range in agreement with the sensibility of the instrument. The spectra recorded at any concentration were mainly coincident, and for this reason, suppression of signal was not considered. The spectra shown in this work are those recorded at 100 μM, which showed better S/N ratio.

Molecular mass determination was performed by electrospray ionization mass spectrometry equipped with a time-of-flight analyzer (ESI-TOF MS) using a Micro Tof-Q Instrument (Bruker Daltonics Gmbh, Bremen, Germany) calibrated with NaI (200 ppm NaI in a 1:1 H_2O:isopropanol mixture), interfaced with an Series 1100 HPLC pump (Agilent Technologies) equipped with an autosampler, both controlled by the Compass Software. The experimental conditions for analyzing the samples were: 20 μL were injected through a PEEK long tube (1.5 m × 0.18 mm i.d.) at 40 μL/min under the following conditions: capillary-counterelectrode voltage, 5.0 kV; desolvation temperature, 90–110 °C; dry gas 6 L/min. Spectra were collected throughout an m/z range from 800 to 2000. The liquid carrier was a 95:5 mixture of 15 mM ammonium acetate and acetonitrile, for the analysis at pH 7.0, and for the analysis at acidic pH, a 95:5 mixture of 5 mM formic acid and acetonitrile adjusted at pH 2.4. All the samples were analyzed at least in duplicate to ensure reproducibility. In all cases, molecular masses were calculated according to the method in [17].

3. Results and discussion

The two terrestrial snails *H. pomatia* and *C. aspersum*, synthesize metal-specific MTs in different cell types and under different physiological requirements [10,11]. In Table 1, the sequences of all the peptides used in this work are compared through their Clustal X alignment [18]. It is worth noting the high similarity (more than 50% for all pairs) among all the sequences and the absolute conservation of their Cys residues (Table 2).

Once the five recombinant Zn–MT complexes had been synthesized, mixtures containing the isoforms of a same organism (*i.e.* Zn–HpCdMT plus Zn–HpCuMT; and Zn–CaCdMT plus Zn–CaCdCuMT and Zn–CaCuMT) at different molar ratios were prepared and analyzed by ESI-TOF MS at neutral and acidic (2.4) pH, to respectively obtain information about the Zn- and apo-forms present in the sample. In all cases, the ESI-MS spectra recorded from 800 to 2000 m/z units were deconvoluted in order to avoid the differences in intensity that can be observed among different charge states of the same mixture.

The spectra obtained for the mixture of *H. pomatia* isoforms (Zn–HpCdMT and Zn–HpCuMT) at neutral pH (Fig. 1) show that HpCdMT renders mainly a major peak, Zn_6–HpCdMT, together with minor signals corresponding to lower nuclearities, while the HpCuMT isoform yields three much more similar peaks identified as the Zn_6–HpCuMT, Zn_5–HpCuMT and Zn_4–HpCuMT species. However, the intensities observed for both Zn_6-species at 1:1 equimolar ratio clearly reveal a higher sensitivity of the Zn–HpCdMT preparation than that of Zn–HpCuMT to be ionized under our working conditions. Hence, when increasing the amount of the HpCuMT isoform, maintaining the concentration of HpCdMT at 50 μM, the intensity of the HpCuMT peak clearly increased but the ratio between both major peaks did not change in accordance with their relative abundance. In fact, it was necessary to raise the HpCuMT: HpCdMT ratio to nearly threefold to achieve the same ESI-MS intensity for both Zn_6-species. Interestingly, the intensity ratios between Zn_6–HpCuMT, Zn_5–HpCuMT and Zn_4–HpCuMT were constant along the mixtures.

Table 2
Analysis of the sequence similarities (identity) among the MT isoforms of each organism studied: *H. pomatia* and *C. aspersum*.

Identity	
Hp**Cu**MT *vs.* Hp**Cd**MT	56.9%
Ca**Cd**MT *vs.* Ca**Cu**MT	51.5%
Ca**Cd**MT *vs.* Ca**CdCu**MT	54.5%
Ca**Cu**MT *vs.* Ca**CdCu**MT	68.7%

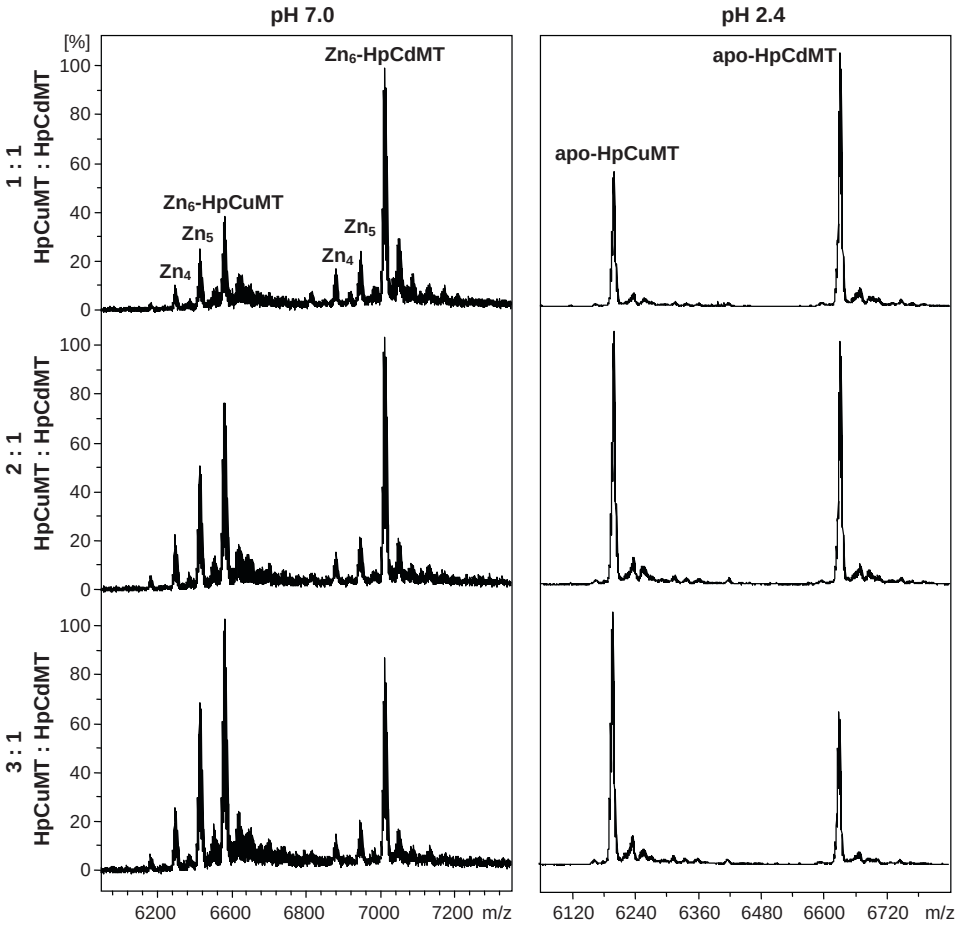

Fig. 1. Deconvoluted ESI-TOF MS spectra recorded both at pH 7.0 and at pH 2.4 for distinct mixtures of the preparations of the recombinant Zn-complexes of the HpCuMT and HpCdMT *Helix pomatia* MT isoforms at the indicated molar ratios. In all cases the starting material where the Zn complexes of the respective isoforms, which at acid pH render the corresponding apo-forms.

The same measurements run at acidic pH (Fig. 1) revealed that the propensity of the apo-HpCdMT peptide to ionization was also higher than that of HpCuMT, but interestingly, here only a 1:2 ratio was required to reach similar ESI-MS peak intensities. Therefore, again the ESI-MS intensity ratios between both apo-forms were not changing in proportion to their relative abundance in the sample.

Overall these results are consistent with a much higher sensitivity to be ionized of the Cd isoform (HpCdMT) of *H. pomatia* than the corresponding Cu isoform (HpCuMT) when analyzing either their Zn- or their apo-forms.

The mixture of the three *C. aspersum* isoform Zn–MT complexes (*i.e.* Zn–CaCdMT, Zn–CaCuMT and Zn–CaCdCuMT) at neutral pH (Fig. 2) informed about the different speciation yielded by each isoform. While Zn_6–CaCdMT was the clearly major species in the Zn–CaCdMT preparation, synthesis of the Cu- and the CdCuMT isoforms as Zn-complexes rendered mixtures of several metallated species. It is worth noting that the differences of the molecular masses of the 3 peptide apo-forms (Table 3) allow perfect identification in the spectra of the respective metal-loaded complexes. If only the major peak of each preparation is considered, it is observed that in the 1:1:1 equimolar mixture, Zn_6–CaCdMT yields a clearly higher intensity than the Zn_6–species of the other two isoforms. Doubling the molar ratios of Zn–CaCuMT and Zn–CaCdCuMT in relation to Zn–CaCdMT, produced a slight increase in the intensities of their peaks, although they were not proportionally doubled. In parallel to the observation for the HpMT isoforms (see above), a threefold CaCuMT:CaCdMT ratio was also required for both Zn_6-species to reach the same signal intensity in the spectra. Also as already observed for *H. pomatia*, the ESI-MS peak intensity ratios rendered by the Zn–MT complexes of the same isoform exhibiting different stoichiometry were constant in all the measurements.

The analysis at acidic pH of the mixtures of Zn–CaMT preparations at different ratios (Fig. 2) revealed that the apo-CaCdCuMT peptide exhibits a higher sensitivity to ionization than the corresponding Zn_6-species, at neutral pH. Thus, at the 1:1:1 equimolar conditions, both apo-CaCdCuMT and apo-CaCdMT showed similar ESI-MS peak intensities. Interestingly, the increase in the amount of CaCdCuMT in the mixture provoked a clear increase in the relative intensity of its signals, which was not as well observed in the case of CaCuMT. Finally apo-CaCuMT yielded the same peak intensity than apo-CaCdMT at a 2:1 molar ratio.

Therefore, and contrarily to the observations for the *H. pomatia* MT isoforms, not all the *C. aspersum* MT isoforms behaved equally as Zn-complexes (neutral) than as apo-peptides (acidic pH ESI-MS measurements), since CaCdCuMT showed a patently higher sensitivity to ionization at pH 2.4 than at pH 7.0.

The data obtained from the ESI-MS analysis of the apo-forms and the Zn-complexes corresponding to two *H. pomatia* and three *C. aspersum* MT isoforms suggest different lines of discussion. First, the ESI-MS ionization sensitivity of each MT peptide appears to vary depending to their metallation state, this is if they are in holo- or apo-form, although each MT isoform behaves differently to this respect. These disparities could be attributed to dissimilarities in their amino acid composition. However, the analysis of the sequence of each MT isoform (Table 3) reveals that they share

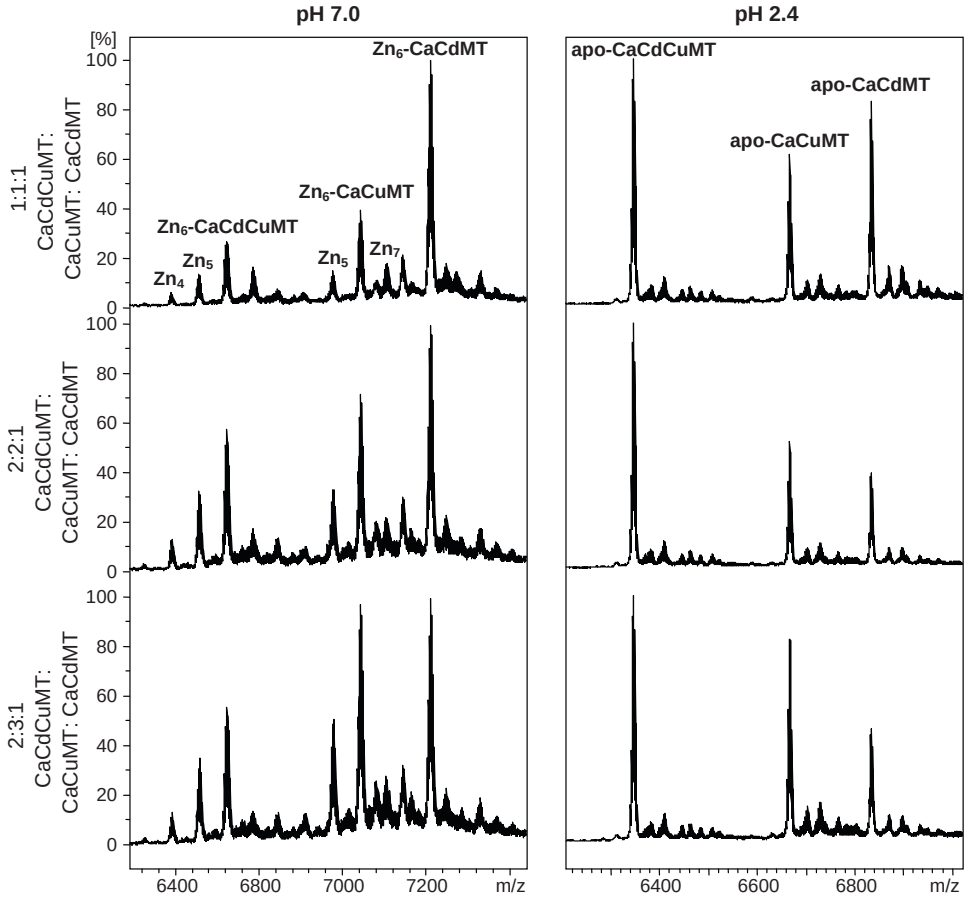

Fig. 2. Deconvoluted ESI-TOF MS spectra recorded both at pH 7.0 and at pH 2.4 for distinct mixtures of the CaCdMT, CaCdCuMT and CaCuMT *Cornu aspersum* MT isoforms at the indicated molar ratios. In all cases the starting material where the Zn complexes of the respective isoforms, which at acid pH render the corresponding apo-forms.

Table 3
Analysis of several features of the polypeptide chain of the different MT isoforms of *H. pomatia* and *C. aspersum* studied.

Protein	Molecular mass	Theoretical pI	Peptide length (C + H)	Polar aa (%)	Apolar aa (%)
Hp**Cu**MT	6261.92	8.13	65 (18 + 1)	16.9 ($4^-/7^+$)	24.6
Hp**Cd**MT	6625.54	8.29	67 (18 + 0)	23.9 ($6^-/10^+$)	20.9
Ca**Cu**MT	6658.29	7.91	67 (17 + 1)	14.9 ($4^-/6^+$)	22.4
Ca**CdCu**MT	6339.06	7.87	66 (18 + 0)	12.1 ($3^-/5^+$)	22.8
Ca**Cd**MT	6824.81	8.29	69 (18 + 0)	23.1 ($6^-/10^+$)	23.2

most of their features, the only significative differences arising from their content in polar amino acids, both acidic and basic ones. The higher percentage of polar amino acids of the HpCdMT sequence, around 24%, would ensure a higher ionization potential than for HpCuMT, at any conditions, as observed in our experiments (Fig. 1). Unfortunately, this reasoning cannot be used when considering the results obtained for CaCdCuMT: this peptide shows the lowest percentage of polar amino acids in its sequence, and it behaves at neutral pH similarly to CaCuMT, which exhibits also low amount of polar amino acids, but, unexpectedly, at acidic pH it shows always the highest ESI-MS intensities among the peaks observed. Consequently, the behaviour of CaCdCuMT does not correlate with the expected behaviour if only considering the amino acid sequence, namely the number of polar amino acids.

On the other hand, it is worthwhile to highlight that the metal–MT species of the same peptide but that differ in their metal content (for example, Zn_6–HpCuMT, Zn_5–HpCuMT and Zn_4–HpCuMT) preserve their relative ESI-MS peak intensities at any of the conditions assayed, thus confirming that the differential behaviour of a metal–MT complex depends on the nature of its peptide sequence more than in the relative metal load. Consequently, the mass data can afford a semi quantitative value for their determination.

4. Conclusions

The overall results allow reaching two main conclusions. First, it can be established that the electrospray ionization process of the MT molecules here studied clearly depends on their metallation state, *i.e.* if the proteins are analyzed at neutral pH (Zn-loaded forms) or at acidic pH (apo-forms).

Second, it can be deduced that every MT protein shows an individual and particular behaviour when ionized at certain conditions. Consequently, the intensities of the ESI-MS signals of distinct MT proteins, still if they are closely related isoforms of the same organism, cannot be directly related with their relative abundances in a sample, and this is correct even when these peptides or metal–MT complexes are analyzed under exactly the same conditions.

In spite of these two conclusions, we have also observed that the relative intensities of the ESI-MS peaks corresponding to differ-

ent species of the same protein that only differ in their metallation degree (metal–MT complexes of different stoichiometry) are maintained through the diverse experiments undertaken. Consequently, we can also state that peak intensities of similar species of the same MT peptide can be related with their relative abundance in the solution.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.talanta.2010.10.060.

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