Detection and identification of rabbit liver metallothionein-2 subisoforms by capillary zone electrophoresis - inductively coupled plasma mass spectrometry and microbore HPLC - electrospray mass spectrometry

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Two recently developed approaches to the subisoform-specific detection and identification of metallothionein (MT) isoforms are compared. They are based on the coupling of capillary electrophoresis with ICP MS and microbore chromatography with electrospray MS. The resolution of HPLC is judged to be slightly better since differently metallated forms of the same sub-isoform can be separated. Detection by electrospray MS is mandatory to avoid the attribution of artefact signals observed in element-specific chromatograms to different sub-isoforms.

Introduction

To date, most of works concerning metallothioneins have been referring either to the MT-fraction (as isolated by gel filtration) or to two MT-isoforms resulting from the further fractionation of the MT-fraction by anion-exchange chromatography into MT-1 (eluting first) and MT-2 (eluting second). However, a further characterization of these fractions by reversed-phase HPLC [1] and capillary zone electrophoresis [2] revealed in each of them the existence of several proteins, termed MT subisoforms, that vary by one or two aminoacids or have extra residues. Since each MT subisoform is known to be expressed by a single gene, the identification and determination of the particular subisoforms may contribute to a better understanding of the

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different MT gene expression rates and of the role and functions of the individual MT-subisoforms. A deeper insight into these phenomena requires more powerful analytical techniques that would combine high selectivity (capability of resolving MTs varying by one aminoacid only) and high sensitivity (femtomolar detection limits are desirable).

The characterization of MT presents several challenges that include the identification of metals involved in the complex, calculation of the stoichiometry of the complex, and the precise (unique aminoacid sequence) identification of the ligand present. This can be achieved by combining three types of approach [3]. They include:

(*i*) sensitive detection of the presence of metal complexes with the individual MT-subisoforms by combining reversed-phase chromatography with ICP MS [4];

(*ii*) on-line determination of the molecular masses of the complexes separated by reversed-phase HPLC [4-6] or CZE [7,8] using electrospray MS. Note that HPLC - ES MS of apo-MT-forms is extremely difficult in contrast to CZE - ES MS;

(*iii*) identification of the particular apo subisoforms by on-line post-column acidification of eluents in HPLC or CZE [9].

This paper focusses primarily on the development and evaluation of an interface allowing the on-line use of an ICP mass spectrometer as detector in CZE to separate metal complexes with the different subisoforms or differently metallated complexes with one subisoform. The results obtained by this technique were compared with those obtained by microbore reversed-phase HPLC with ES MS detection. An example of MT-2 purified from rabbit liver was used.

Experimental

Experiments were carried out with a Beckmann P/ACE 2200 (Beckmann Instruments, Inc., Fullerto, CA) and a HP 4500 ICP mass spectrometer (Hewlett-Packard-Yokagawa, Yamanashi-Ken, Japan). A microconcentric nebulizer (MicroMist) (Glass Expansion, Romainmotier, Switzerland) was used for the interface. It was mounted on the standard Scott spray chamber. The concept was similar to that described by Lu *et al.* [10] The capillary was threaded through the colinear end of a T-piece; on the exit end it was inserted into a stainless steel tube and further sheathed with Teflon tubing to the base of the nebulizer. The capillary was inserted into the central tube of the nebulizer. The sheath flow (5 mM NH4NO3 containing 100 ng ml^{-1} yttrium at 20 µl min^{-1}) was added through the bottom arm of the T. The electrical circuit was achieved by grounding the stainless steel tube.

Electrospray MS data were acquired using a PE-SCIEX API 300 pneumatically-assisted electrospray (Ion-spray®) triple-quadrupole mass spectrometer (Thornhill, ON, Canada). The MT-2 subisoforms were separated on a Vydac C_8 150 mm × 1 mm × 5 µm column. An ABI 140C microbore syringe pump, an ABI Model 112A injection module and an ABI Model 785A absorbance detector equipped with a microbore cell (Applied Biosystems, Foster City, CA, USA) were used. The elution was realised with a linear gradient of acetonitrile (10 – 16 %B) within 50 min at 40 µl min^{-1}. Buffer A was 5 mM acetate buffer in water (pH 6.0) and buffer B - 5 mM acetate buffer (pH 6.0) in water-acetonitrile (50:50, v/v). The eluate was acidified on line with a mixture (30:70, v/v) of formic acid and methanol that, supplied at 4 µl min^{-1} allowed a final pH of 1.9 to be obtained and the conversion of Cd, Zn and mixed Cd-Zn complexes to apo-MT-subisoforms. Copper complexes, if present would not be completely demetallated under these conditions.

The preparation analyzed, rabbit liver MT-2 (34H95161) was purchased from Sigma-Aldrich (Saint-Quentin-Fallavier, France).

Characterization of the MT polymorphism by CZE - ICP MS

Although CZE (with UV detection) was shown to obtain excellent resolution of MT subisoforms [2], the maintenance of this resolution when an ICP mass spectrometer is used as detector is difficult. The principal reasons include the appearance of the laminar flow through the capillary as a result of the nebulizer suction and the need (unless a sheathless interface is used) for a makeup flow at the level exceeding 10 - 100 times the electroosmotic flow to establish the electrical contact and to assure the optimum nebulization [10-12].

The optimization of the system aimed at the maximum resolution of the MT subisoforms. The initial operational conditions (buffer, voltage, injection mode) had been adopted from the CZE with UV detection optimized earlier. The pH of the running buffer was set to 6 or 7 to ensure the presence of the fully metallated (Cd, Zn) metallothioneins. The optimization of the interface focussed on the elimination of the laminar flow (to increase the resolution) and the optimization of the nebulizer efficiency (to increase the sensitivity). Since both these parameters are dependent mainly on the nebulizer gas and liquid sheath flows a compromise needed to be achieved. The possibility to limit the laminar flow by applying negative pressure during separation was also attempted.

An electrophoregram obtained at the optimized separation conditions for a purified rabbit liver MT-2 preparation is shown in figure 1. The number and intensity of peaks vary as a function of the metal determined which suggests the coexistence of different complexes of one MT-isoform or the presence of different isoforms having different affinities to the different metals. The major peak (the most intense signal in the Cd- and Zn-electrophoregram, peak 4) is likely to

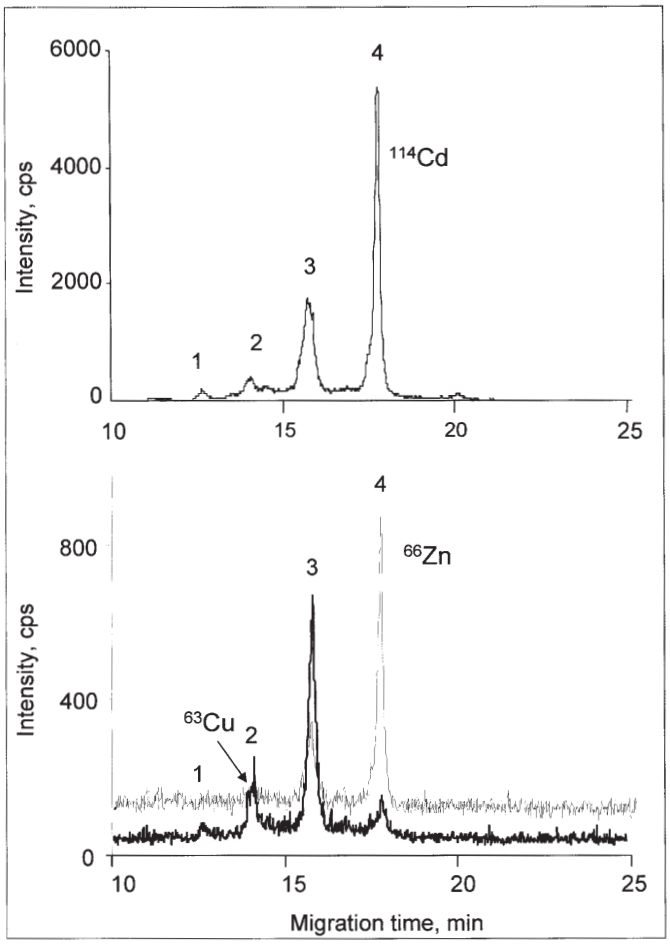

Figure 1. Characterization of a rabbit liver MT-2 preparation by capillary zone electrophoresis with ICP MS detection. Run buffer: 5 mM ammonium acetate (pH 6.0); capillary length: 80 cm (50 μm i.d.); applied voltage: 20 kV; injection: electrokinetical at 3 kV for 5 s. The peak identification is impossible at this stage due to the lack of authentic MT-2 subisoform standards.

correspond to a mixed $(Cd,Zn)_7$-MT-2 complex; the presence of copper is insignificant. In the second most intense peak (peak 3) the contribution of Cu exceeds that of Zn and is comparable with that of Cd indicating a mixed (Cd, Cu, $Zn)_x$-MT-2 complex. Peaks 1 and 2 correspond to mixed $(Cd,Cu)_x$-MT-2 complexes. Whereas in the peak 1 cadmium dominates, roughly equimolar Cd and Cu contributions seem to be present in the second peak.

The use of the element selective detection in CZE allows one to raise a question mark regarding the attribution of different peaks in CZE-UV electrophoregrams to different MT-subisoforms. Should it be proven that different metal complexes of the same isoform can be separated by CZE, the use of a molecule specific detector will be essential to study the MT genes expression. The coupling CZE - ES MS being not available yet in our laboratory, some data obtained by microbore reversed phase HPLC with ES MS detection for the same MT-2 preparation are shown below.

Characterization of the MT polymorphism by microbore reversed-phase HPLC - ES MS

A chromatogram obtained under the optimum conditions is shown in figure 2. It shows a similar morphology that the electrophoregram in figure 1 with the exception that the major peak is split in two (peaks 4 and 5). After acidification of the chromatographic eluent, the peak 4 and peak 5 give the identical mass spectrum corresponding to the MT-2a subisoform (calculated Mr 6124.5 ± 0.5, 6125.3 theoretical). This indicates that the originally present complexes of the same subisoform with the different Cd, Zn stoichiometry can be separated by reversed-phase chromatography and may lead to artefact peaks when element-specific or UV-detection is used. Peak 3 (Mr = 6084.5) is likely to correspond to the non-acetylated MT-2a. Peak 2 (Mr 6144.5 may be tentatively attibuted to MT-1a (theoretical Mr 6145.3). We are unable to eveoke a hypothesis regarding the identity of peak 1.

Assuming that the elution order in CZE and reversed-phase HPLC is the same molecular masses can be attributed to the apo-subisoforms producing peaks 1-3. Note that the ES mass spectrum taken at the apex of peak 2 that contains a significant proportion of Cu shows two signals at the +4 ionization state, the second probably corresponding to a Cu-MT-1a complex.

Conclusions

The high resolution achieved by microbore column HPLC and CZE makes these techniques attractive for studying the distribution of metal complexes with the different MT iso- and subisoforms. ICP MS is an element-specific detection technique allowing a sensitive detection of metals bound to metallothioneins but its use with CZE requires a custom-designed interface. Electrospray MS is the primary technique to identify the peaks, but post-column acidification is necessary to increase the sensitivity and the precision of the molecular mass determination. This post-column acidification is readily achievable using sheath flow with acidic buffer. An alternative would be the use of high-resolution MS. The couplings CZE-ES MS and reversed-phase HPLC - ICP MS of which the development is underway should bring complementary information for the characterization of MT subisoforms and their metal-binding.

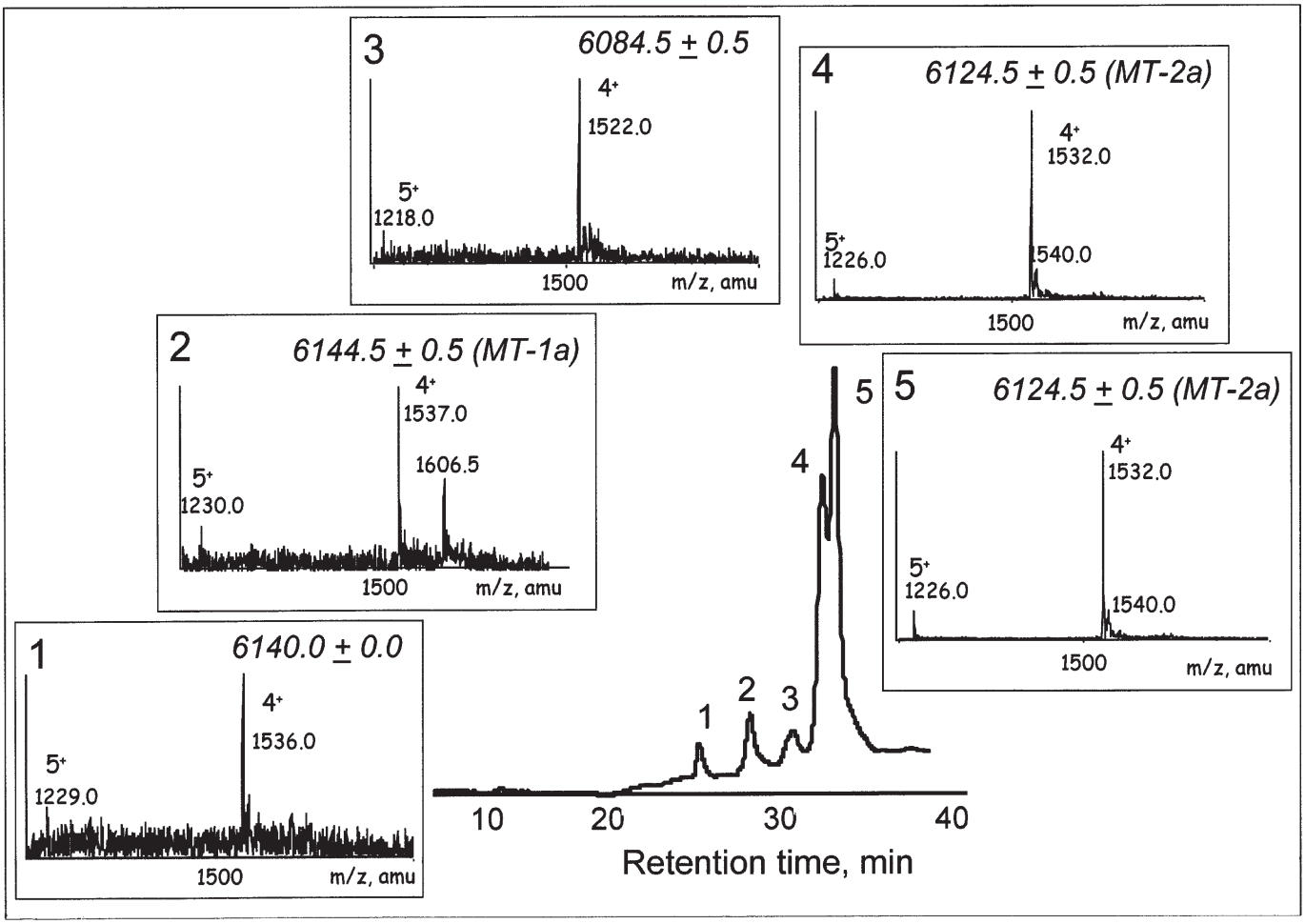

Figure 2. Characterization of a rabbit liver MT-2 preparation by reversed-phase microbore HPLC with electrospray MS detection. Conditions given in the text. The insets show electrospray MS spectra registered at the apex of each of the peaks 1-5.

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