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Talanta 69 (2006) 988–995
Talanta
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Investigation of zinc binding metallothioneins' polymerization in tris(hydroxymethyl)-aminomethane buffer by coupling of size exclusion chromatography with electrospray ionization mass spectrometry

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Received 24 July 2005; received in revised form 27 November 2005; accepted 2 December 2005
Available online 3 March 2006

Abstract

Polymerization of metallothioneins (MTs) is one of the commonly encountered puzzles in researching the structure and function of metallothioneins. In this work, a method involving SEC coupled with negative ion electrospray ionization mass spectrometry (ESI-MS) detection has been developed for the study of zinc binding MTs' polymerization in tris(hydroxymethyl)-aminomethane (TRIS) acetate buffer at physiological pH. This hyphenated technique allows separating the different polymeric states of MTs by SEC, followed by on-line identification of the individual MT subisoforms in each polymeric peak by ESI-MS detection. Purified MT subisoforms (MT-2d and MT-2a), MT-2d and MT-2a mixture and rabbit liver MT complexes were investigated in the experiments to confirm the results obtained. From the results, both oxidative polymerization and non-oxidative oligomerization were found. The cystein-dependent oxidation results in the tetrameric peak as shown in the chromatograms of oxidized MT-2d, and stable dimeric and monomeric of MT were detected in this peak by MS. For the dimeric and trimeric peaks, different MT subisoforms were detected. In the five major subisoforms detected in rabbit liver MT complexes, MT-2a and MT-2c exist primarily as trimer, while MT-2e, MT-2d and MT-1a exist mainly as dimer. Our results suggest that in the three kinds of polymers, dimer, trimer and tetramer that were found in samples, the tetramer comes from the oxidation of MT molecular; for the dimer and trimer resulting from cystein independent oligomerization, they are closely associated with the charge of subisoform.

Keywords: Polymerization; Size-exclusion chromatography; Electrospray ionization mass spectrometry; Metallothioneins; Tris(hydroxymethyl)-aminomethane

1. Introduction

Metallothionein (MT) is a cysteine-rich, low-molecular-weight intracellular protein with a high affinity for metals such as zinc, copper, cadmium and mercury. It was first isolated 40 years ago from equine renal cortex, and was shown structurally to contain 61 amino acids, of which 20 were cysteine residues [1]. These proteins are ubiquitously distributed in the cytoplasm of animals, plants, and microbes. MTs possess two domains of metal-mercapto clusters and have a very special absorption spectrum [2]. The roles of MT have been extensively investigated in the homeostasis regulation of essential metals including zinc (Zn) and copper (Cu), the detoxification of potentially toxic heavy metals such as cadmium (Cd) and mercury (Hg), and the protective actions against various oxidative stress conditions [3–6].

Polymerization of MTs is one of the commonly encountered puzzles during the process of research into their structure and function. A series of papers have been published in the literature concerning the study of polymerization of MTs using nuclear magnetic resonance (NMR), X-ray, circular dichroism (CD), size-exclusion chromatography, capillary zone electrophoresis

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doi:10.1016/j.talanta.2005.12.002

(CZE) and mass spectrometry techniques [7–15], but the exact mechanism is still unclear. Palumaa and co-workers studied the effect of Cd on Cd7–MT-2 and found two additional Cd ions per monomer and increased metal–metal exchange in the β domain [8,9]. The formation of intermolecular S–Cd–S bonds was proposed to be the reason for this non-oxidative dimerization in MTs. On the other hand, some papers had characterized stable dimers of MTs [10–12,14]. Since in all cases the oligomerization of the species could be reversed by treatment with 2-mercaptoethanol or dithiothreitol (DTT), an oxidative mechanism for these processes was concluded by these authors. Other than the view of cystein-dependent polymerization that was usually discussed, Hou and his co-workers in their recent study also proposed a cysteine-independent polymerization mechanism. Based on their experimental results and theoretical calculations, a molecular recognition mechanism of metallothioneins' polymerization in solutions was proposed [15].

Mass spectrometry (MS) has recently emerged as the principal technology of choice to determine protein sequence, as well as to identify non-covalently bound constituents of protein complexes [16–19]. The capability of ESI-MS to analyze metal binding properties of mammalian MTs has been well demonstrated in literature studies [20–23]. Size-exclusion chromatography (SEC) is also a well-established separation method for the study of MT polymerization [8,14]. However, SEC with traditional UV detection is incapable of identifying the species detected, and consequently, off-line sequencing techniques are often necessary to characterize the eluate. The on-line coupling of SEC with ESI-MS for the studying of MTs' polymerization is expected to provide distinct information to recognize the different polymeric states and online identification of the individual MT subisoforms in polymeric peaks. Furthermore, the coupled system would eliminate the extensive sample manipulation and time consuming sample cleanup step, thus reducing potential source of contamination and change during the treatment process (usually caused by oxidation).

Despite the benefits, no study on coupling SEC to ESI-MS for the analysis of MTs' polymerization could be found in the literature to the best of our knowledge. In fact, only few reports are available on the subject of direct ESI-MS analysis [14,24]. Our objective in this study was to improve the technique of coupling SEC to ESI-MS in order to effectively obtain information on the polymerization of MTs. It is well known that the composition of the mobile-phase used in the system is crucial for a successful ESI-MS analysis because the response of analytes by ESI-MS is significantly affected by the type of modifier used [25,26]. Ammonium acetate buffer is commonly used for the detection of MTs by ESI-MS performed in positive mode [21,27–29]. However, the buffer system was ineffective to attain a sensitive MS detection of MT species [30,31], and could even disable their analysis. Recently, we had developed a more sensitive method for MT identification by negative ion ESI-MS in Tris(hydroxymethyl)-aminomethane (TRIS) acetate buffer [32], which is popularly used to maintain a physiologic condition in vitro. Thus, TRIS acetate at physiologic pH (7.4) was used as mobile phase in this experiment.

2. Experimental

2.1. Apparatus

HPLC analysis was carried out on an Agilent model 1100 system equipped with a UV diode array detector (DAD) (Agilent, USA). Injection was performed by using model 7725 injection valve with a 20 μl injection loop (Rheodyne, CA, USA). Electrospray MS experiments were performed using 1100 MSD pneumatically assisted electrospray octopole-quadrupole mass spectrometer (Agilent, USA). The Agilent Chemstation software was used for calculating the molecular masses and deconvolution of protein mass spectra.

2.2. Reagents and standards

Acetonitrile was of LC grade (Merck, German). TRIS, acetic acid were of high purity grade (Alfa Aesar, USA). MT preparation isolated from rabbit liver containing a mixture of two isoforms, MT-1 and MT-2, was purchased from Hunan Lugu Biotechnology (China). Water, purified by a Nanopure Diamond (Barnstead, USA) water purification system, was used to prepare all solutions. The water used was sparged with argon gas to remove dissolved oxygen to attain a non-oxidizing environment. Buffer (20 mM) used for SEC was prepared with TRIS, and the pH was adjusted to 7.4 with acetic acid. The rabbit liver MT solution was prepared by dissolving 5 mg of MT preparations in 2 ml of water. The concentration of the total MTs was measured by Ellman's assay [33], and 1.9 mg ml^{-1} of the proteins was detected. The solution was kept in the fridge at −80 °C in the dark. Working solutions were prepared by the dilution of the stock solution. Unless otherwise stated, protein elution was performed in 20 mM TRIS acetate buffer, pH 7.4. Rabbit liver MT-2a and MT-2d were purified by reversed-phase high-pressure liquid chromatography. Briefly, the two isoforms were purified on a Zorbax 300SB C18 150 mm × 2.1 mm, 5 μm column with particle pore size of 300 Å (Agilent, USA). The pump flow was set at 0.25 ml min^{-1}. Buffer A was 10 mmol l^{-1} TRIS buffer in water (pH 7.4), and buffer B was acetonitrile. Metallothionein complexes were eluted with a linear gradient from 5% B to 10% B within 50 min, and the eluted fractions of the two proteins (at 17.5 min and 20.1 min) were collected. After lyophilization, the fractions containing the two proteins were desalted by centrifugation using 3 kDa cut-off centrifugal ultrafiltration cartridge (Millipore, USA), and then diluted with water. The concentrations of the proteins were measured by Ellman's assay [33], 1.4 mg ml^{-1} of MT-2a and 1.1 mg ml^{-1} of MT-2d were detected. The solution was then kept at −80 °C as stock solution. Working solutions were prepared by the dilution of the stock solution with 20 mM TRIS acetate buffer (pH 7.4).

For the experiment to evaluate polymerization by oxidation, part of purified MT-2d preparation was prepared and put into air environment for three days at room temperature as oxidized MT-2d preparation. After oxidation, a fraction of oxidized preparation was loaded and added with 0.1% (w/w) dithiothreitol (DTT) to evaluate the effect of DTT on the oxidized MT species.

These two preparation solutions were then kept at 4 °C in the dark for analysis.

2.3. HPLC conditions

A commercial high-performance G-3000 PWxl SEC column (TSK, Japan) of 300 mm × 7.8 mm i.d. and a particle size of 6 μm was used. Separation was performed at a flow rate of 0.5 ml min^{-1} with an eluent consisting of 20 mM TRIS acetate (pH 7.4) at a column temperature of 25 °C. Molecular weights were determined on the column calibrated with bovine albumin (66,400 Da), lysozyme (14,300 Da), pancreatic trypsin inhibitor (BPTI) (6500), bovine insulin chain B (3500) and cobalamin (355). All were obtained from Sigma–Aldrich Corp. (USA).

2.4. Electrospray MS conditions

The flow out of the SEC was added with 10% of acetonitrile (v/v) by a "T" connector to stabilize the MS signal, and then split with approximately 0.20 ml min^{-1} flow directed into the ESI source by a micro-splitter (UPCHURCH, USA). MS data for Zn binding MT complexes were acquired in negative ion mode by scanning over the *m*/*z* ranges of 700–1800 to gain 4–8 charge state of the proteins. For the detection of apo-MT complexes, ESI-MS was performed in positive ion mode with the *m*/*z* ranges of 700–1800. The heated capillary temperature was set at 300 °C. The collision induced dissociation (CID) voltage was set to 100 V. The electrospray voltage (ESV) for positive ionization and negative ionization were 4000 V and 3500 V, respectively. The mass spectra were acquired with a step size of 0.15 u.

3. Results and discussion

MTs' functional conformations are supposed to be different kinds of polymers, i.e., dimers, trimers, or tetramers in vivo [34]. Certain MTs have been shown to exist as isoforms and subisoforms that are the products of genetic polymorphism [35]. Isoforms and subisoforms with different amino acid composition have different isoelectric points and different hydrophobicities, hence they would form different polymerized states in solutions. The methods usually used for the studies of MT's polymerization such as circular dichroism, dynamic light-scattering, or NMR are unable to recognize MT isoforms or subisoforms in their different polymeric states without pre-purification. ESI-MS with the capability of identifying MT subisoforms would facilitate these studies to gain insight into the MT polymerization of MT complexes.

For the coupling of SEC to ESI-MS, buffer with a certain concentration of inorganic additive (such as 10–50 mM) is required to maintain a certain pH and to reduce ionic interactions with the column material. Based on our precious research [32], high MS response of MT could be obtained in TRIS acetate buffer even at a concentration of 20 mM under neutral condition. Thus 20 mM TRIS acetate buffer at a physiologic pH (pH 7.4) was chosen as the mobile phase and dilution solution for MT preparations. Rabbit liver MTs was chosen as they are by far the best-described MTs in the literature and commercially available of preparations. Preparations abundant in zinc and with minimum contents of copper were used to reduce the number of species potentially present in the system studied. Two purified MT subisoform (MT-2a and MT-2d), their mixture, and rabbit liver MT complexes were used in the experiments to confirm the result obtained.

3.1. Identification of MT isoforms in purified preparations and rabbit liver MT complexes

ESI-MS was employed to identify MT isoforms in the two chromatographically isolated preparations (MT-2a and MT-2d) and rabbit liver MT complexes, to be used for further study. The identification was performed in positive ionization mode in acidic condition (1% formic acid) under which Zn is dissociated from the MT molecular, giving rise to an apo-MT in the analyte solution. Fig. 1 shows the ESI-MS data for the three preparations. As can be seen from the figures, the protonated species are distributed across 4+, 5+, 6+, 7+ and 8+ ions. For the two purified preparations shown in Fig. 1a and b, individual MT isoform, respectively corresponding to the molecular mass of 6125 (Fig. 1a) and 6215 (Fig. 1b) were found in each preparation. The detected molecular masses for the two isolated isoforms are identical with the calculated ones on the

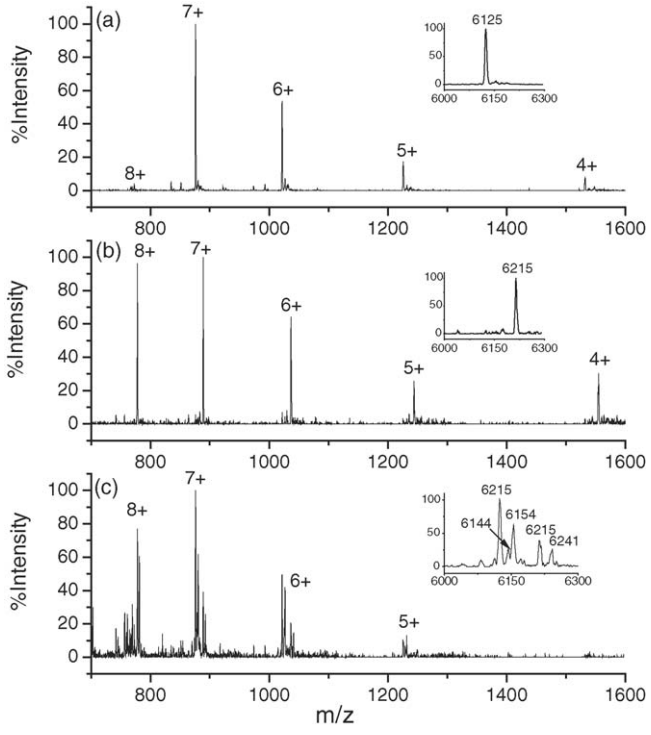

Fig. 1. The positive ion ESI-MS spectra for MT-2d, MT-2a and rabbit liver MT complexes. The mass spectra obtained by feeding an aqueous solution containing 1% (v/v) formic acid into the source at the rate of 0.2 ml min^{-1}: (a) MT-2a (Mr 6125, measured 6125), 0.70 μg of sample was injected; (b) MT-2d (Mr 6215, measured 6215), 0.55 μg of sample was injected; (c) rabbit liver MT complexes, the five major subisoforms detected (from left to right) were MT-2a, MT-1a (Mr 6145, measured 6144), MT-2c (Mr 6155, measured 6154), MT-2d and MT-2e (Mr 6242, measured 6241), 0.95 μg of sample was injected.

basis of the published sequence of MT-2a and MT-2d (6125 and 6215). For rabbit liver MT complexes as shown in Fig. 1c, five major putative subisoforms were detected by the method, and four of them were easily recognized as MT-2a (6125), MT-2c (6155), MT-2d (6215) and MT-2e (6241) according to the reported reference [22,30]. However, it was hard to distinguish MT-1a and MT-2b since their masses are too close (6145 and 6146) to resolve with the instrument used. Anion exchange chromatography was introduced to distinguish the two isoforms, and the result showed that the mass of 6144 detected in this analyzed preparation belonged to MT-1a as we showed in another paper [36].

There are several minor peaks on the left of the intact protein peaks from *m/z* 700 to 1000 in the mass spectrum as shown in Fig. 1a. These fragment ions are postulated to have produced from the loss of *N*-acetylated Met and Asp residues from the amino terminal of MT [20].

3.2. Calibration curve of SEC

The molecular mass of MT species could be obtained when the SEC column is calibrated by standard materials with known molecular weight, which were eluted under the same buffer conditions as the MT sample. The molecular weight calculated as denary logarithms versus the retention time (t, min) of each peak was plotted. The linear equation was:

$$\log M = -0.3046t + 8.2983$$

The regression coefficient (R^2) was 0.9907. This calibration procedure provides an estimation of the retention time of different polymeric states of MT. As the molecular mass of zinc binding MT is about 6600, the tetramer, trimer, dimer and monomer would be eluted approximately at 12.7 min, 13.1 min, 13.7 min and 14.7 min, respectively.

3.3. Oxidative polymerization of MT detected by UV

MT samples stored under aerobic conditions are supposed to form aggregates, even in the absence of excess metals [10]. In the experiments, a fraction of purified MT-2d preparation was put into air environment for three days at room temperature to produce oxidized MT-2d preparation. Part of the oxidized MT-2d preparation was then added with 0.1% dithiothreitol (DTT) to evaluate the effect of DTT on the oxidized MT species. MT-2d, oxidized MT-2d and oxidized MT-2d in the presence of 0.1% DTT were assessed on an analytical SEC column. The chromatograms for the three preparations with UV detection are shown in Fig. 2. As can be seen from Fig. 2a, one major peak at 12.6 min which is corresponding to dimer is observed in MT-2d preparation. As for the oxidized MT-2d, besides the dimeric peak as that found in Fig. 2a, one visible peak at 13.6 min which is corresponding to tetramer is also observed in Fig. 2b. Considering that MT species under aerobic conditions were reported to readily form aggregates by oxidation [10–12], the tetramer found only in oxidized MT-2d may be a product of oxidation. For the oxidized MT-2d in the presence of 0.1% DTT, the chromatogram obtained (Fig. 2c) is similar to that of MT-2d

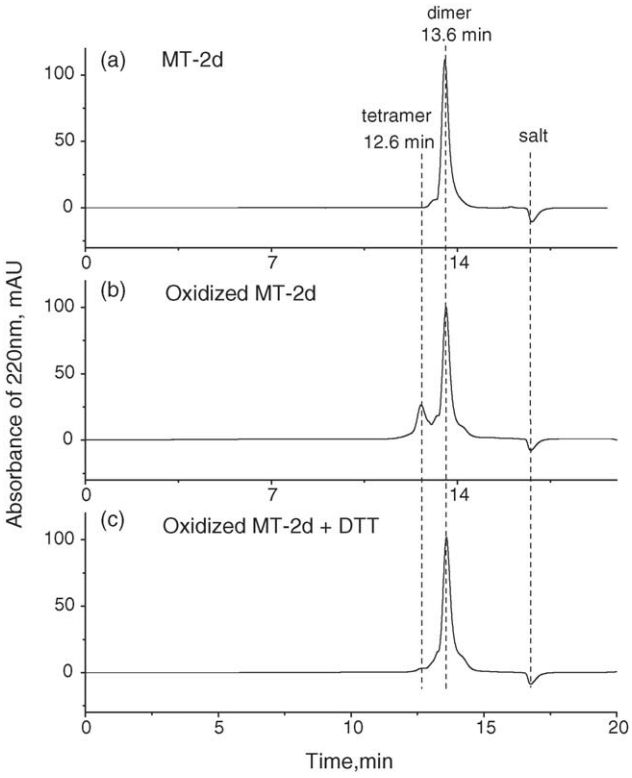

Fig. 2. Chromatograms of MT-2d (a), oxidized MT-2d (b) and oxidized MT-2d in the presence of 0.1% dithiothreitol (c) preparations by SEC with ultraviolet detection. Separation was performed at a flow rate of 0.5 ml min^{-1} with an eluent consisting of 20 mM TRIS acetate (pH 7.4) at a column temperature of 25 °C; 20 μl of MT fractions at a concentration of 0.11 mg ml^{-1} was injected.

shown in Fig. 2a. It seems that the DTT added in the oxidative MT-2d does significantly reduce and resume the MT species which were oxidized as seen in oxidized MT-2d preparation. This finding confirms that an oxidative mechanism is reasonable to explain the formation of tetramer found in oxidized MT-2d.

From the results, there are two polymers detected in the preparations used. The tetramer shown in chromatograms is likely a product of oxidation, and could be reduced back to its original form by treating with DTT. For the dimer, it seems to be independent of oxidation. The unique polymeric state could be formed by a mechanism related to their physiological functions [15].

3.4. Polymerization of MT-2d and oxidized MT-2d detected by SEC-ESI-MS

The total ion chromatograms (TICs) and mass spectra obtained for the oxidized MT-2d preparation and MT-2d preparation are shown in Fig. 3. The TICs obtained are consistent with those obtained by UV detection. For oxidized MT-2d preparation, Fig. 3a shows one major peak corresponding to dimer and one visible peak corresponding to tetramer. As to MT-2d preparation, one major peak corresponding to dimer is observed in Fig. 3b. The mass spectra of tetramer and dimer are presented in Fig. 3c–d.

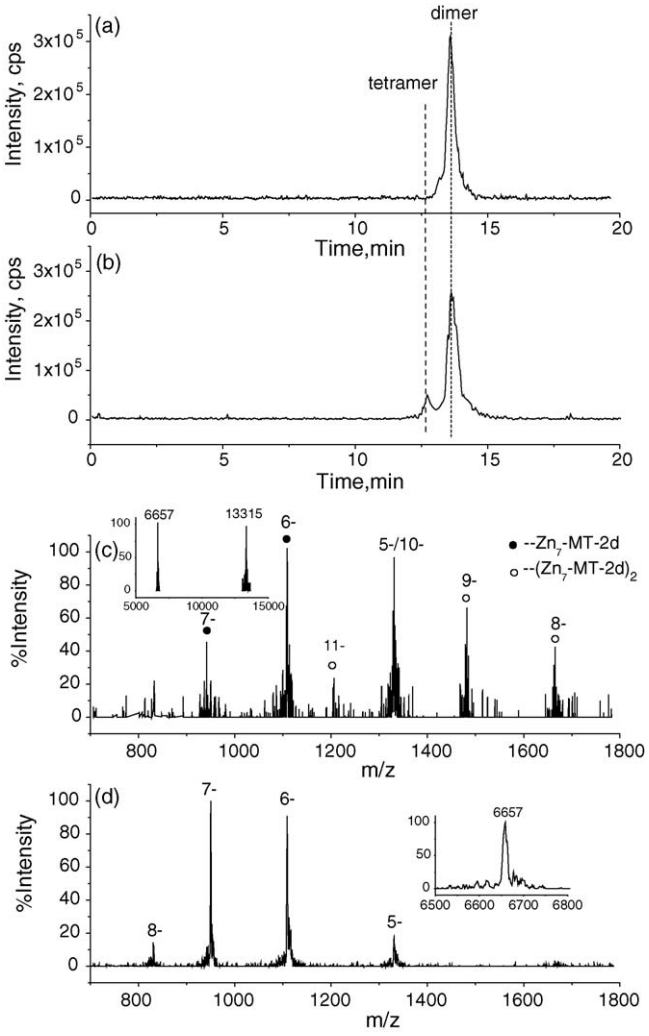

Fig. 3. Total ion chromatograms (TICs) and mass spectra of MT-2d preparation and oxidized MT-2d preparation by SEC coupled with negative ion ESI-MS detection. The separation conditions are shown in Fig. 2; the flow out of the SEC was split with approximately 0.20 ml min^{-1} flow directed into the ESI source: (a) TIC of MT-2d preparation; (b) TIC of oxidized MT-2d preparation; (c) mass spectrum of tetramer shown in (b), Zn7-MT-2d (Mr 6659, measured 6657) and dimeric Zn7-MT-2d (Mr 13,318, measured 13,315) were detected; (d) mass spectrum of dimer shown in (a) and (b), Zn7-MT-2d (Mr 6659, measured 6657) was detected.

Zn7-MT is well known to be the predominant form of the protein in the normal adult mammal [37]. Fig. 3d shows the mass spectrum of dimer, the major oligomerization state shown in chromatograms. The spectrum contains one envelope of ionization states from 4 to 8, and an outstanding mass of 6657 is deconvoluted from the raw ESI-MS data. This measured mass is consistent with the expected molecular mass for a neutral species of Zn7-MT-2d (6658, apoMT + 7Zn − 14H), and no significant signals resulting from MT species with a different number of attached Zn ions could be found. It seems that Zn7-MT-2d is the unique compound detected in the dimeric peak. The finding suggests that the dimeric state of purified MT-2d preparation in solution is primarily resulted from the interaction between Zn7-MT-2d molecules. Actually, the relative weak interactions such as steric complementarity, hydrophobic and electrostatic interaction between MT molecules are supposed for the formation of non-oxidative oligomers [15]. These weak interactions in the solution phase could be dissociated in the gas phase under the condition, and thus resulting in the detection of monomeric mass of MT-2d. For the tetramer shown in Fig. 3b, the mass spectrum shown in Fig. 3c contains two envelops of ionization states, one consists of 8-, 9-, 10- and 11-charge states corresponding to dimeric molecular mass of Zn7-MT-2d (13,315), while the other exhibits 4-, 5-, 6- and 7-charge states of the monomeric one as that observed in the dimeric peak (Fig. 3c). The stable dimer detected by MS could be a product of covalent interaction between MT molecules by oxidation, as no such compounds were found in the dimeric peak under the same condition. The finding of oxidative dimer seems to be consistent with that obtained by Zangger and Armitage, who proposed in their recent research that the formation of a disulfide bond involving Cys-36 was responsible for the formation of oxidative polymers in mammalian MTs [38]. Besides the dimeric Zn7-MT-2d, significant amount of monomeric Zn7-MT-2d, which was detected in the dimeric peak, was also found in this peak. The MS data suggests that the tetrameric peak is a coordinate interaction of oxidative and non-oxidative MT-2d moleculars. This result could be explained by the model involving polymerization in solutions. As found for dimeric peak, non-oxidative MT-2d was shown to readily form dimer in TRIS solution. For the oxidative dimer, each MT-2d molecular region on the dimer would remain its capability of binding with another MT-2d molecular, thus leading to the formation of tetramer. From this study, both oxidative and non-oxidative polymers were detected. For the tetramer determined in oxidized MT-2d preparation, the stable oxidative dimer of MT-2d and MT-2d molecules detected by MS is coordinate for the formation of tetramer. For the dimer, the substantial amount of Zn7-MT-2d detected can be contributed to its formation.

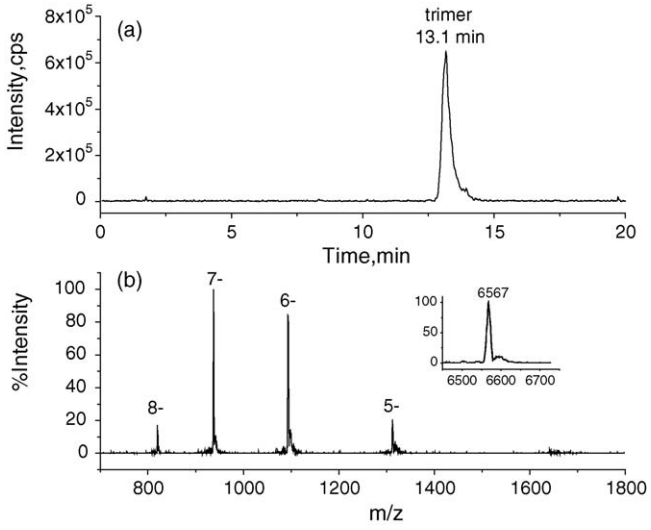

Fig. 4. Total ion chromatogram (TIC) and mass spectrum of purified MT-2a preparation by SEC coupled with negative ion ESI-MS detection. The separation conditions are shown in Figs. 2 and 3; 20 μl of MT fractions at a concentration of 0.14 mg ml^{-1} was injected: (a) TIC; (b) mass spectrum of trimer shown in (a), Zn7-MT-2a (Mr 6569, measured 6567) was detected.

3.5. Oligomerization of purified MT-2a detected by SEC-ESI-MS

The TIC of the purified MT-2a preparation by ESI-MS detection is shown in Fig. 4a. From the chromatogram, one major peak corresponding to trimer is observed. The mass spectrum of trimeric peak is shown in Fig. 4b. Similar to that found for Zn7-MT-2d, five negatively charged states (4−, 5−, 6−, 7− and 8−) of the protein are observable in the spectrum. A well-resolved *m/z* signal corresponding to mass 6567 was deconvoluted from the raw mass data. The measured mass compares well with the expected molecular mass of Zn7-MT-2a (6568). Compared with MT-2d, MT-2a is shown to be preferable to form trimer in TRIS acetate buffer.

3.6. Oligomerization of MT-2d and MT-2a mixture detected by SEC-ESI-MS

As the results obtained above, individual MT-2d or MT-2a was shown to preserve their particular polymeric states as dimer

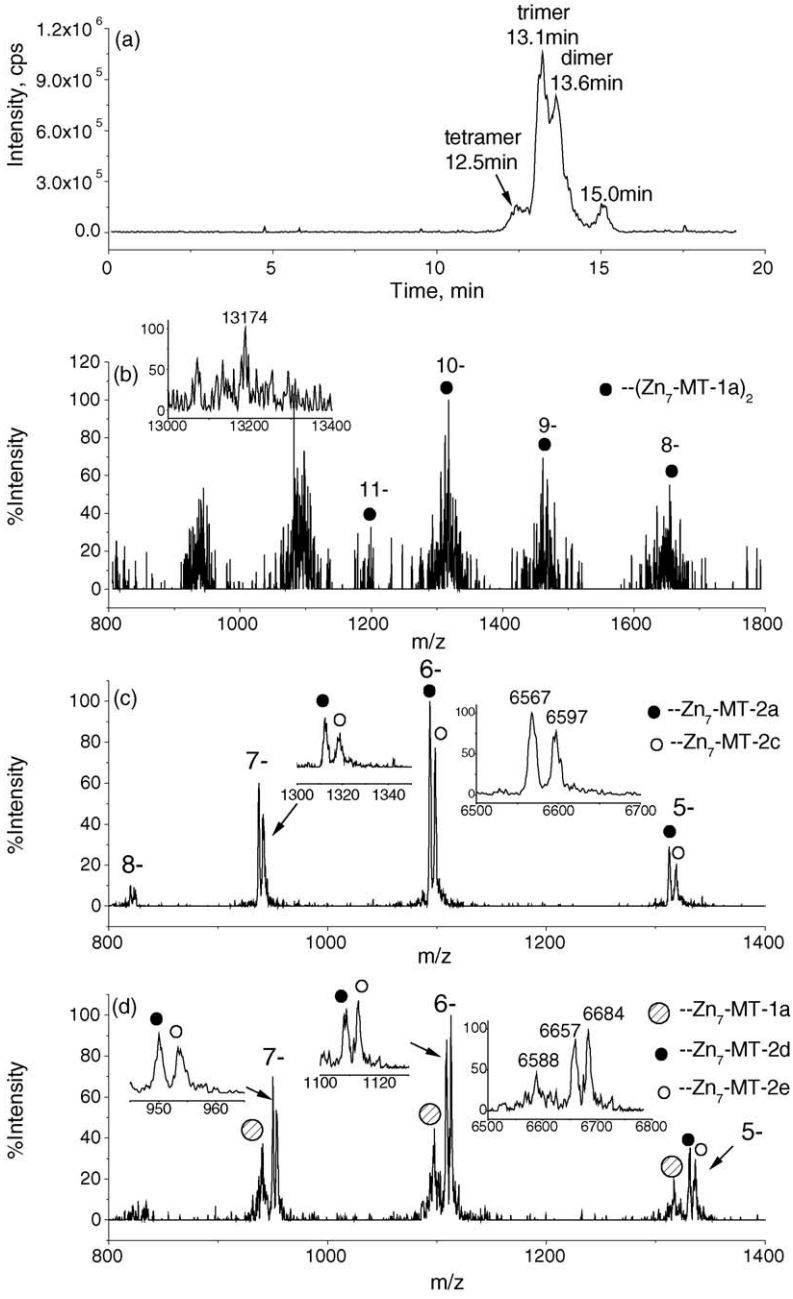

Fig. 5. Total ion chromatogram (TIC) and mass spectra of rabbit liver MT complexes by SEC coupled with negative ion ESI-MS detection. The separation conditions are shown in Fig. 2; 20 μl of MT fractions at a concentration of 0.19 mg ml^{-1} was injected: (a) TIC; (b) mass spectrum of tetramer shown in (a), the filled circles denote ions representing dimeric Zn7-MT-1a (13174); (c) mass spectrum of trimer shown in (a), Zn7-MT-2a (the filled circles denote ions, 6567 measured) and Zn7-MT-2c (the open circles denote ions, 6597 measured) were detected; (d) mass spectrum of dimer shown in (a), Zn7-MT-1a (the shaded circles denote ions, 6588 measured), Zn7-MT-2d (the filled circles denote ions, 6657 measured) and Zn7-MT-2e (the open circles denote ions, 6684 measured) were detected.

or trimer in solution. To further identify the oligomerization by different MT subisoforms, MT-2d and MT-2a were mixed together as MT-2d and MT-2a mixture, and then the preparation was analyzed by the coupling system. Two visible peaks, which respectively accord with the retention times of MT trimer and dimmer, were found from the chromatograms (data not shown). ESI-MS results showed that MT-2d were found in the apexes of the trimeric peak, while MT-2a was detected in dimeric peaks, as that shown in Figs. 3d and 4b. It seems that MT-2d and MT-2a still preserve their particular oligomeric states in solutions even as a mixture. Actually, the different electrostatic interactions are supposed to be vital in the formation of MT oligomer [15]. Dimer is supposed to readily form by the electrostatic attractions between MT monomer. For the formation of trimer, strong hydrophobic interactions and relative weak electrostatic repulsions between the monomer and dimer would be required. The presence of different oligomerization states by MT-2d and MT-2a in solution is somewhat surprising since these two MT species both belong to MT-2, which may have the slight differences of hydrophobic interactions and electrostatic interactions. Actually, the classification of the mammalian MT subisoforms as the type MT-1 or MT-2 is not based on their charge [39,40]. A detailed study of MT amino acidic primary sequences shows that MT-2d has a similar charge as MT-1a [41]. As the formation of stable trimers of MT-1 is difficult because of the relatively strong electrostatic repulsions [15], the finding of MT-2d in dimeric peak is rational. For MT-2a, the electrostatic repulsions between monomers and dimers in the trimers are much weaker, so trimer would form.

3.7. Polymerization of rabbit liver MT complexes detected by SEC-ESI-MS

The usefulness of SEC-ESI-MS in the analysis of two chromatographically isolated MT subisoforms was clearly demonstrated in the analysis of purified preparations as stated above. We next employed this approach in the analysis of more complicated rabbit liver MT complexes.

The chromatogram for rabbit liver MT complexes with ESI-MS detection is shown in Fig. 5a. There are three visible peaks in the chromatogram, corresponding to dimer, trimer and tetramer. Beside the three polymeric peaks, one distinct peak (at about 15 min) is observed in Fig. 5a. As no distinct mass peak corresponding to MT species were found, it could be an impurity in the commercial MT preparations. The mass spectra of the different polymers are shown in Fig. 5b–d. For the tetrameric peak shown in the chromatograms, the mass spectrum in Fig. 5b shows a similar pattern to the one found in oxidized MT-2d preparation. As described above, the tetramer detected is a product of MTs' oxidation which would form during purification and storage. The complicated *m*/*z* signals of each ion cluster from the mass spectrum indicate a complicated oxidation process involving MT molecules. A significant envelop of peaks corresponding to dimeric molecular mass of Zn7-MT-1a (13,174) was deconvoluted from the raw mass data. Distinct identification of the other peaks requires a mass spectrometer of higher accuracy and sensitivity such as TOF mass analyzer. As for the mass spectrum of trimeric peak shown in Fig. 5c, it shows two distinct envelops of mass peaks, which respectively correspond to the mass of 6567 and 6597. The masses detected are identical to that of Zn7-MT-2a and Zn7-MT-2c. The MS data suggests that they are MT-2a and MT-2c that mainly contribute to the form of the trimeric peak shown in chromatogram. This finding is consistent with the result obtained from the two purified MT preparations. Since MT-2a and MT-2c are the classic MT-2 isoforms that are different in only one amino acid residue, MT-2c may have the similar oligomeric state as that of MT-2a. Thus both MT-2a and MT-2c could be found in the trimeric peak. The mass spectrum of dimeric peak is shown in Fig. 5d. Three distinguishable MT species with the mass of 6684, 6657 and 6588 were found from the mass spectrum. The determined masses are respectively consistent to the molecular mass of Zn7-MT-2e, Zn7-MT-2d and Zn7-MT-1a. The result suggests MT-2e, MT-2b and MT-2d are more favorable for the formation of dimers than MT-2a and MT-2c. Although MT-2e and MT-2d both belong to MT-2, they have similar charge as MT-1a [41]. The relatively strong electrostatic repulsions between the dimer and monomer make the formation of stable trimer difficult, thus they were detected in dimeric peak.

In summary, both oxidative polymer and non-oxidative oligomer were found in rabbit liver MT complexes preparation. For the tetramer, which has been proven to result from oxidation, rather complicated *m*/*z* signals were detected. Unambiguous MS identification of these compounds in the peak calls for a higher accuracy and sensitivity mass spectrometer. For the non-oxidative oligomers, the oligomeric states are shown to be closely associated with the charge on MT subisoforms in the complexes. In the five major isoforms found in rabbit liver MT complexes, MT-2a and MT-2c are found to exist preferably as trimer species, while MT-2e, MT-2d and MT-1a prefer the formation of dimers in TRIS acetate buffer.

4. Conclusions

In conclusion, we have developed a method by coupling SEC with on-line ESI-MS detection for the investigation of the polymerization of zinc binding metallothioneins' in TRIS acetate buffer. The SEC separation allows the separation and differentiation among three polymeric states, namely, tetramer, trimer and dimer; and the ESI-MS detection provides distinct information for the recognition of MT subisoforms in these polymeric peaks. Our results show that both oxidative polymerization and non-oxidative oligomerization of MT could be found in the preparations investigated. For the oxidative polymerization, the process leads to the formation of tetramer species. For non-oxidative oligomerization, different MT subisoforms seem to preserve their particular oligomeric states in solution. In the five major isoforms detected in rabbit liver MTs, MT-2a and MT-2c prefer the formation of trimer species, while MT-2e, MT-2d and MT-1a prefer the formation of dimers.

Acknowledgements

The authors thank Su-Ge Lu, Miao Jing and Xiao-Qin Xu for their kind help. This work was financially supported by the Key

Project of the National Natural Science Foundation of China (20175022).

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