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A sensitive negative-ion electrospray ionization mass spectrometry detection for metallothionein in tris(hydroxymethyl)aminomethane acetate buffer

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Abstract

Although positive-ion (PI) electrospray ionisation mass spectrometry (ESI-MS) has been usually applied for the analysis of native metallothioneins (MT) isoforms binding with metal ions, it suffers from the lack of insensitivity under neural conditions because of the low efficiency of protonation during ESI process. In this study, multiply deprotonated metallothionein (Zn_7-MT-2a), produced from tris(hydroxymethyl)aminomethane (TRIS) acetate solutions under near neutral condition, was analyzed by negative-ion (NI) ESI-MS. Compared with the ammonium acetate buffer system which has been normally used for ESI-MS experiments, the use of TRIS acetate buffer results in the formation of more abundant ions and higher charge states of MT-2a, and consequently higher intensity is attained. The sensitivity enhancement of the system could be explained by the high gas-phase proton affinity, small molecular volume of the anion (acetate), and the high hydrogen consumption by TRIS in the buffer system, which would all favor the deprotonation of the analyte during NI-ESI process. Factors that may affect the NI-ESI responses, such as acid added in buffer, pH and concentration of TRIS acetate had been evaluated, and the result showed that using acetic acid, lower pH under the pH range evaluated, higher concentration of TRIS acetate all favored the MS detection of MT-2a. Our finding sheds light on a buffer system that may offer substantial sensitivity advantages in the studying of weakly bound, non-covalent complexes such as metal binding MTs, which are usually analyzed under near neutral conditions.

Keywords: Positive-ion; Negative-ion; Electrospray ionization mass spectrometry; Tris(hydroxymethyl)aminomethane; Metallothionein

1. Introduction

From the initial pioneering development of electrospray ionization (ESI) by Fenn et al. [1], electrospray ionization mass spectrometry (ESI-MS) has been employed successfully to investigate various non-covalently bound protein complexes including specific protein–metal ion complexes [2]. Generally, ESI-MS analysis of polypeptides and proteins has been performed in the positive-ion (PI) mode. The basic amino acid residues (arginine, lysine, histidine, and the NH_2-terminus) have sufficiently large dissociation constants to ensure that the target proteins are most likely being highly charged (protonated) under acidic conditions. Although acidic pH (often 0.1–1% formic or acetic acid) are most commonly used for peptide and protein analysis by PI-ESI because of their optimal sensitivity, such condition is not generally tolerated for non-covalently bound protein complexes because they would be denatured in a solution with pH value outside of the neutral (6–8) range [2]. Thus, the adoption of experimental systems at higher pH toward the neutral, physiological regime is encouraged for studying non-covalent protein interactions [3]. Negative-ion (NI)-ESI has also been applied for polypeptide analysis [4–6], but rel-

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atively fewer studies of polypeptides have been reported. The pH value used at NI-ESI experiments is typically 8–10, which is closer to neutral condition. It seems that NI-ESI may be a desirable tool for studying non-covalent protein complexes [4,6–9].

Metallothioneins (MTs) are a group of cysteine-rich, low-molecular mass intracellular protein with a high affinity for metals such as zinc, cadmium, and mercury. They play an important role in the homeostasis regulation of essential metals, the detoxification of potentially toxic heavy metals, and the body's therapeutic and protective actions against many disease states under oxidative stress conditions [10–12]. The capability of ESI-MS to analyze MTs was first demonstrated by Yu et al. [13], and since then the technique has been applied to investigate the metal binding properties of mammalian MT [14–16]. Since the equilibrium of complex formation between MT and metal is strongly pH-dependent, the control of pH in solution is crucial for the detection of the intact protein [17]. Under acidic conditions, the analysis of the complex would be impractical because the links between the metal and the ligands in the complex would be destroyed. There are many reports concerning the identification of MTs by PI-ESI-MS detection in ammonium acetate buffer (5–10 mM) under neutral pH [14–16,18,19]. However, ESI-MS detection under such conditions suffers from the lack of sensitivity, poor signal-to-noise ratio [20].

It appears that most of the ESI-MS studies on protein folding reported in the literature have been conducted under conventional polarity conditions. For example, the unfolding of basic or neutral proteins is commonly studied in the PI mode, whereas for folding studies on acidic proteins the NI mode is often used. For the case of native zinc or cadmium binding MTs, the coordination of Cd^{2+} or Zn^{2+} ions into the protein results in a net increase of 6 negative charges on the protein (7 bivalent metal ions replace 20 H^+) [21]. Hence, the detection of MT by NI-ESI-MS was proposed in this paper. Tris(hydroxymethyl)-aminomethane (TRIS) buffer is popularly used to maintain a physiologic condition in vivo, but relatively few reports concerning MS detection of proteins in the buffer could be found in the literature. The present study was designed to investigate the potential of NI-ESI-MS for the analysis of MT isoform in TRIS buffer. The most striking discovery was that the native proteins, which are characterized by low ionization efficiency in PI-ESI, exhibited drastically increased signal intensities in NI-ESI in TRIS acetate buffer under neutral condition. The factors that may affect the NI-ESI responses, including acid added in buffer, pH, and TRIS acetate concentration were discussed herein.

2. Experimental section

2.1. Apparatus

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

TRIS, ammonium acetate, acetic acid, trifluoroacetic acid (TFA), and formic acid were of high purity grade (Alfa Aesar, USA). Hydrochloric acid used was of high purity grade (Peking Reagent Factory, 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, thus to attain a non-oxidizing environment.

MT preparations isolated from rabbit liver by size-exclusion chromatography were purchased from Hunan Lugu Biotechnology (China). The preparations rich in zinc and with minimum contents of Cu were chosen to reduce the number of species potentially present in the system due to the limited MS resolution of our instrument. Rabbit liver MT-2a was purified from MT preparations by reverse-phase high-pressure liquid chromatography. Briefly, the protein was purified on a Zorbax 300SB C_{18} 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 to 10% B within 50 min, and the eluented MT-2a fraction (at 19 min) was collected. After lyophilization, the protein was desalted by centrifugation using 3 kDa cut-off centrifugal ultrafiltration cartridge (Millipore, USA). The concentration of the protein was measured by Ellman's assay [22], and 2.5 mg mL^{-1} of the protein was detected. The solution was then kept at −4 °C as stock solution. Working solutions were prepared by the dilution of the stock solution with buffer as required.

2.3. Electrospray MS conditions

Injection was performed by using Model 7725 injection valve with 5 μL injection loop (Rheodyne, CA, USA). With a syringe pump (Agilent, USA) infusing acetonitrile/water (10:90, v/v) (for blank control) at a flow rate of 0.2 mL min^{-1}, the instrument was equilibrated until the ESI response of three consecutive injections became stable. All measurements were performed in triplicate. MS data for Zn binding MT-2a with PI-ESI detection was acquired by scanning over the *m/z* ranges of 1000–2300 for 3+ to 6+ charge state ions, whereas 800–1800 was scaned for for 4− to 7− charge state ions in NI-ESI. The heated capillary temperature was set at 300 °C. The collision induced dissociation (CID) voltage was 100 V. The electrospray voltages (ESV) of PI-ESI and NI-ESI were both set to 4000 V. The mass spectra were acquired with a step size of 0.15 u.

3. Results and discussion

NI-ESI-MS has been used to study the specific interactions between metal ions and their respective metal binding proteins [7–9]. Those involving the MS analysis of MTs, however, have mostly been performed in the PI-ESI mode, and to the best of our knowledge, no study on NI-ESI-MS analysis of MTs has been reported in the literature. Our initiation to carry out the investigation on the use of NI-ESI-MS for MT analysis in TRIS buffer was prompted by two considerations. The first was the consideration that the complexes between MTs with cadmium and/or zinc ions are negatively charged at neural pH, and would thus be more amenable to the NI-MS detection. The second was to find an effective buffer system to maintain a neutral condition and for a sensitive NI-MS detection for MTs. Buffers beyond ammonium acetate and ammonium bicarbonate, which are popularly used, would be taken into consideration since they were found to ineffective to the MS detection [20,23]. TRIS buffer is popularly used to maintain a physiologic condition, which is crucial in the studying of weakly bound, non-covalent complexes. However, few reports concerning the effect of TRIS buffer on MS detection of proteins could be found. In this paper, TRIS buffer was chosen and evaluated for its performance in ESI-MS detection of MT-2a.

3.1. Identification of apo-MT-2a

ESI-MS was employed to identify the purity of MT-2a that was to be used for further study. The identification was performed in positive ionization mode in acidic condition (1%, v/v, formic acid) under which Zn is dissociated from the MT molecular, giving rise to the presence of an apo-MT in the analyte solution. Fig. 1 shows the ESI-MS spectrum of the chromatographically isolated MT isoform. As can be seen from the figure, one major protonated specie and a minor one distribute across the 4+, 5+, 6+, and 7+ ions, respectively, corresponding to the molecular masses of 6125 and 6154. The molecular mass of the major compound (6125 detected) is identical to the calculated one on the basis of the published sequence of MT-2a (6125). The other mass corresponding to the minor compound (6154 detected) is in agreement with that of MT-2c. The trace of MT-2c found in this purified fraction is likely due to the incompletely separation of MT-2c and MT-2a under the condition.

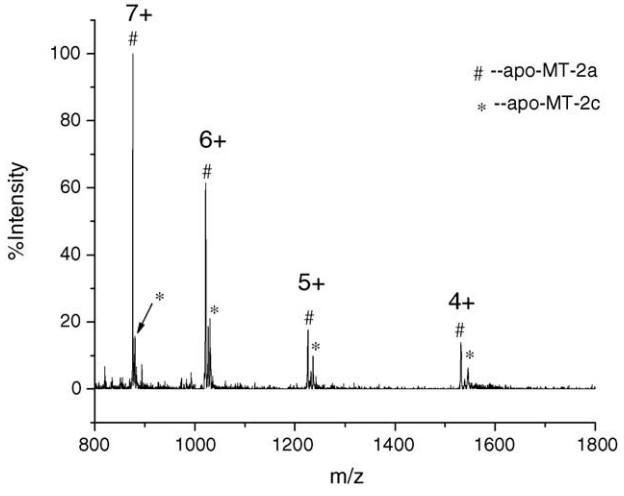

Fig. 1. The ESI-MS spectrum for 1.0 μg of purified MT-2a sample. Two MT isoforms, MT-2a (M_r 6125, measured 6125) and MT-2c (M_r 6155, measured 6154), were detected. The mass spectrum obtained by feeding an aqueous solution containing 1% (v/v) formic acid into the source at the rate of 0.2 mL min^{-1}.

3.2. PI-ESI and NI-ESI mass spectra of MT-2a in ammonium acetate buffer

To date, volatile buffers, such as ammonium acetate and ammonium bicarbonate, are the most popular choices for ESI-MS experiments. For the analysis of MT species, ammonium acetate buffer is usually used for the PI-ESI-MS detection of the proteins. The mass spectrum of the positively ionized MT carrying seven zinc ions is shown in Fig. 2. The figure shows two envelopes of ions corresponding, respectively, to ionization states (4+ and 3+). An outstanding mass of 6567 was deconvoluted from the raw ESI-MS spectrum. This measured mass is in agreement with the expected molecular weight for a neutral species of Zn_7-MT-2a (6568, apo-MT + 7Zn − 14H). The poor signal-to-noise ratio observed reflects the poor detection sensitivity under such condition.

Operating the ESI source in the NI mode leads to multiple negatively charged protein ions via proton abstraction [4]. Although NI-ESI-MS has been used to study the specific interactions between metal ions and their respective metal binding proteins, no reports concerning about MTs by NI-ESI-MS detection had been found from literature. The NI-ESI-MS detection of MT-2a under the same condition by PI-ESI-MS in ammonium acetate buffer had been performed in this study, and the result is shown in Fig. 2b. Similar to that found by PI-ESI detection which shows 3+ and 4+ charge states, two negatively charged states (5− and 4−) of the protein are observable in the spectrum. The mass detected (6566) is in agreement with the mass detected by PI-ESI mode. Furthermore, the NI-ESI detection under such condition is found to increase the MS response to some extent (about 1.5 times) compared to PI-ESI mode. However, the improvement of MS response for MT under the condition is still rather limited, and this leaves a lot to be desired for developing a more sensitive ESI-MS detection method for the proteins.

3.3. NI-ESI mass spectra of MT-2a in TRIS buffer

From an experimental point of view, the keys for a "successful" ESI-MS analysis include maintaining proper solution conditions for keeping the protein complex in its folded, native state and efficient, and effective desolvation of the ESI-generated droplets [3]. TRIS buffers are one of the commonly buffers used by biochemists to control pH in the physiological

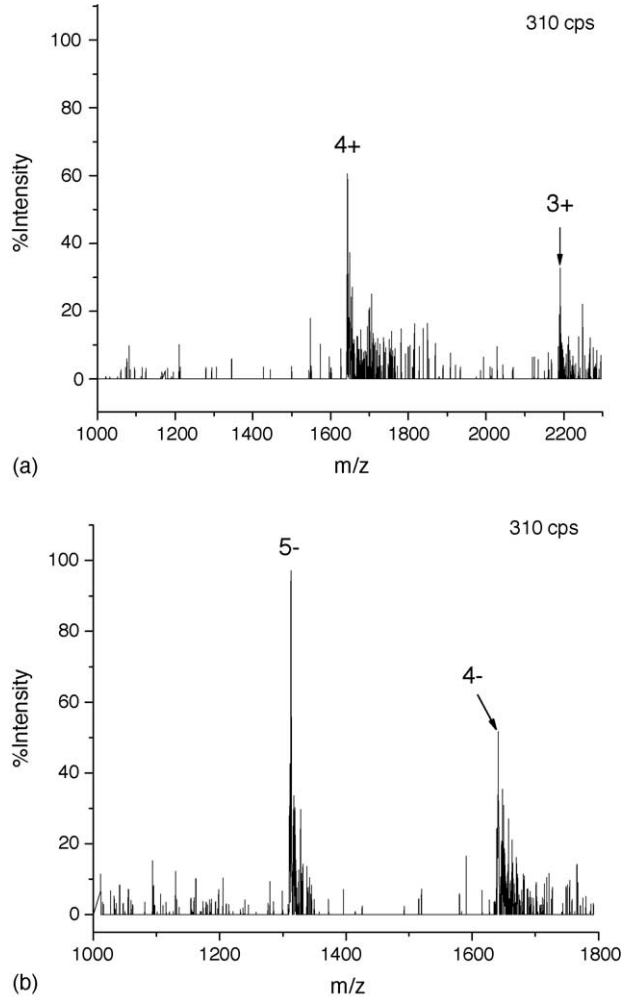

Fig. 2. The ESI-MS spectrum for 1.0 μg of purified MT-2a sample. The mass spectrum obtained by feeding an aqueous solution containing 10 mM ammonium acetate into the source at the rate of 0.2 mL min^{-1}. (a) Positive-ion mode, pH 7.4 and (b) negative-ion mode, pH 7.4. Acetic acid or ammonia was used for the pH adjustment.

range, but TRIS had been found to suppress MS responses in MT analysis by Pawlak et al. [24]. The concern was later confirmed by our experiments. Both PI-ESI and NI-ESI analyses of MT-2a in TRIS hydrochloride buffer were found to produce only very weak signals. It is known that the response of analyte by ESI-MS is a dynamic interplay between the properties of the analyte and mobile-phase modifier [25–27]. For the point of ESI-MS detection, TRIS hydrochloride buffer is not an ideal system, since the hydrochloric acid added would suppress the ionization reactions during the electrospray process due to the high conductivity and the high surface tension [28]. Recently, Wu et al. had found that weak acids significantly increased the NI-ESI responses of four model compounds, and acetic acid was suggested as an ideal acid additive for NI-ESI-MS analysis [27]. Based on the idea, acetic acid instead of hydrochloric acid was used in our experiments to adjust the pH to the near neutral range in the TRIS buffer system. The mass spectrum of MT-2a in 10 mM TRIS acetate

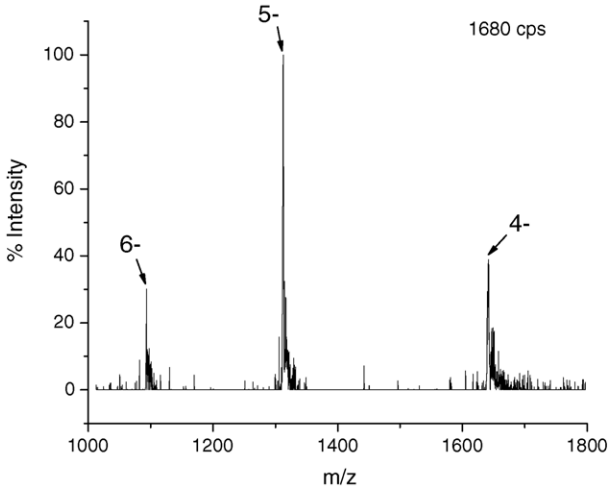

Fig. 3. The ESI-MS spectrum for 1.0 μg of purified MT-2a sample at pH 7.4. The mass spectrum obtained by feeding an aqueous solution containing 10 mM TRIS acetate into the source at the rate of 0.2 mL min^{-1}. Acetic acid was used for the pH adjustment.

solution at pH 7.4 is shown in Fig. 3. Surprisingly, the NI-ESI detection exhibits a more intense signal-to-noise, i.e., about nine-fold increase in signal intensity compared to the PI-ESI analysis, as is illustrated in Fig. 3. The spectrum shows three envelopes of peaks ranging from 800 to 1800 *m/z*, which corresponds to 4−, 5−, and 6− charge states of the protein. The mass detected (6566) is also in agreement with the mass for a neutral species of Zn_7-MT-2a as determined above in ammonium acetate buffer. It should be noted that TRIS buffer was reported to readily form reversible and irreversible adducts through reactions with chemical groups, such as aldehydes and epoxide [29–31]. It is somewhat surprising that no visible TRIS adducts signal was detected from the MS data. The lack of stable TRIS adducts may be due to the absence of active groups, such as aldehydes and epoxide group on MT-2a. Furthermore, the pH value (pH 7.4) at which most of the TRIS in buffer is protonated would also weaken the nucleophilic function of the amino group on TRIS, thus reducing the formation of TRIS adducts by amino group [31]. Other non-covalent adducts, such as anionic (acetate) adduct, were not observed yet. It seems that the non-covalent interaction between these ions and the analyte molecule is not sufficiently strong, and the attaching ions would be lost at the CID voltage (100 V) [32].

As Fig. 3 shows, unlike TRIS hydrochloride buffer, which was found to seriously suppress the ESI-MS response, TRIS acetate buffer gives substantially greater sensitivity enhancement in MS detection. The results suggest that the addition of acetic acid instead of hydrochloric acid in TRIS buffer does provide improvement in MS response. The enhancement in sensitivity is likely due to the high gas-phase proton affinity and the small molecular volume of acetic anion, which would facilitate the deprotonation of the analyte [26]. Besides the anions, the cations in solution would also have a significant effect on MS response. Comparing TRIS acetate run with

ammonia acetate run, more and higher charge states are obtained when using TRIS acetate, as well as higher intensity is attained. Since the two buffers contain approximate concentration of the same anion (acetate), the different response may be due to the presence of different cations in each system. At pH 7.4, ammonium is the major cation in ammonium acetate buffer, while TRIS (protonated) is the primary cation in TRIS acetate buffer [33]. The much higher response of MT-2a in TRIS acetate buffer indicates that TRIS is far effective for a sensitive detection of MT-2a compared to ammonium. The TRIS enhancement of signal intensity had also been found by Huang et al. when they studied the formation of oligonucleotide ion formation [34,35]. From their results, TRIS was effective to enhance signal intensity by consuming hydrogen released from the oligonucleotide and shift the chemical equilibrium towards formation of the oligonucleotide ions. It is known that MT converts to a negatively charged ion by releasing protons in a liquid phase [21]. The high hydrogen consumption mechanism by TRIS seems to be rational to explain the enhancement of signal intensity and the shift to higher negative charge state of MT-2a as observed in our results. However, a number of factors appear to work together in the mechanism of electrospray [25,26], and more thorough investigation is necessary to understand the mechanism better.

While TRIS acetate buffer under neutral condition have greatly favored the NI-ESI detection of MT-2a, it did significantly suppress the PI-ESI response of the protein in our experiment. Our attempt to detect of MT-2a by PI-ESI under the same condition in TRIS acetate buffer could not be achieved as no distinct signals corresponding to MT-2a was found (data not shown). Actually, rather strong $(TRIS + H)^+$ signal was detected when we lowered the *m*/*z* scan range. The result indicates that TRIS is far more competitive than MT-2a for the positive ionization during electrospray process, and would thus significantly suppress the formation of positive MT-2a ion in the ESI source.

3.4. Effect of acid additive on NI-ESI response

Four acid additives that are commonly used in buffer: formic acid, acetic acid, TFA, and hydrochloric acid were chosen and evaluated for their performance on NI-ESI response of MT-2a. The acids were added individually to each sample containing 10 mM TRIS buffer to adjust the pH to 7.4. Fig. 4a shows the responses of MT-2a in these buffers. Comparing the four additives, hydrochloric acid and TFA suppress completely the analyte signal. Actually, TFA and hydrochloric acid were reported to significantly suppress the ionization reactions during the electrospray process due to the high conductivity and the high surface tension of the acids [28,36]. For the relatively weaker formic acid and acetic acid, significantly increase in NI-ESI responses was observed. The result suggests weak carboxylic acids other than the strong acid would be more effective to increase NI-ESI response of MT-2a during MS analysis. Such signal enhancement could be due to the

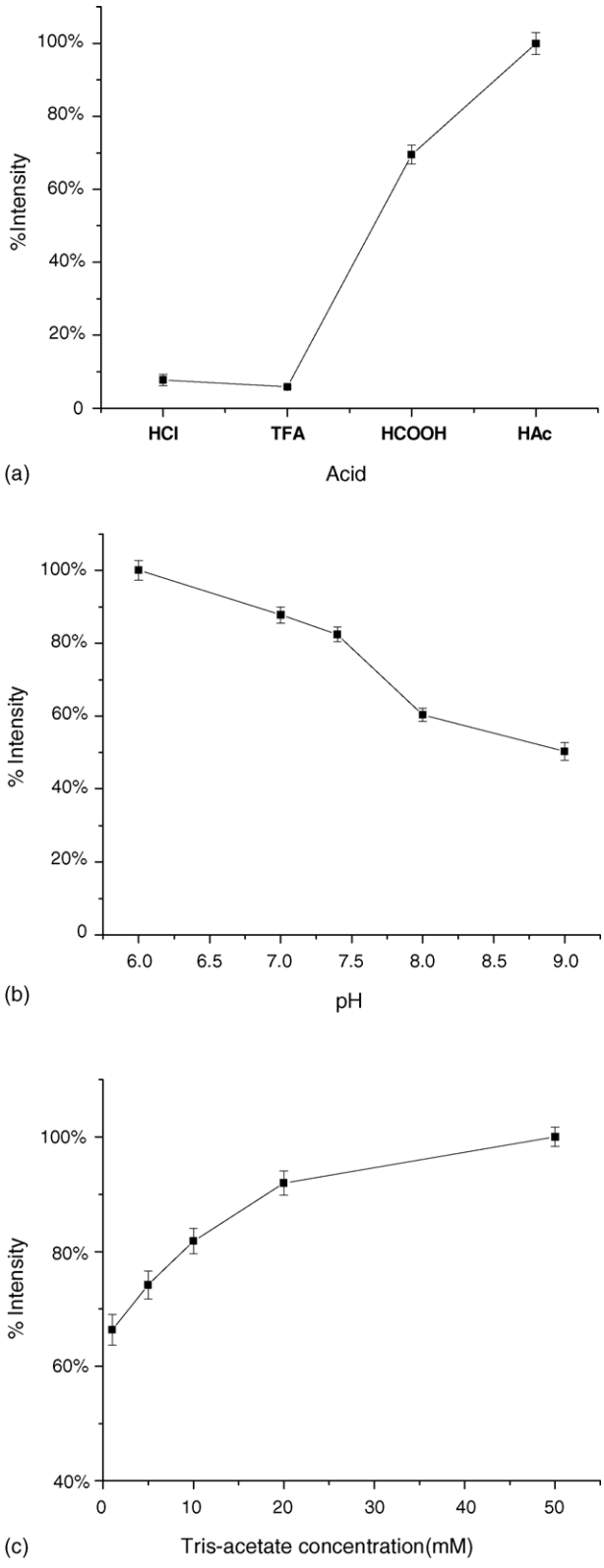

Fig. 4. Effects of different factors on the NI-ESI responses of MT-2a. (a) Acid, at pH 7.4, in 10 mM TRIS buffer; (b) pH, in 10 mM TRIS buffer, acetic acid was used for the pH adjustment; (c) TRIS acetate concentration, at pH 7.4, acetic acid was used for the pH adjustment. The vertical axis represents the mean (±S.D., $N = 3$) ratio of peak area of MT-2a in the presence of modifier, multiplied by 100%.

higher gas-phase proton affinity and small molecular volume of these acid molecules, both factors are known to be beneficial to the ESI process [27]. Compared with formic acid, acetic acid shows even higher sensitivity than that of formic acid, with about 30% increase in signal intensity. This finding is consistent with the report by Sánchez-Rabaneda et al., which showed that formic acid solutions were less effective than acetic acid solutions in their attempt to enhance the NI-ESI response of phenolic compounds [37]. From the results, acetic acid is the most effective additive for the detection of Zn_7-MT-2a by NI-ESI-MS detection. On the other hand, TFA and hydrochloric acid seriously suppress the MS detection.

3.5. Effect of pH on NI-ESI response

The effects of pH from 6.0 to 9.0 in the near neutral range were evaluated in samples buffered by 10 mM TRIS acetate solution. Fig. 4b shows the NI-ESI responses of MT-2a at different pH. The signal response of the protein showed a decreasing trend with increasing pH. About 50% reduction in signal intensity was observed as the pH goes from 6.0 to 9.0. The effect could be explained by that the increased acetate concentration caused by the addition of acetic acid to lower the pH, would greatly facilitate the deprotonation of the analyte, thus increasing the MS response. On the other side, pH may also affect the charges state of the analyte in solution. Higher pH would favor the formation of the deprotonated analyte. However, such effect was not found under the pH range evaluated. The response of MT-2a seemed to be positively related to the concentration of acetate added. Considering the significant decrease of ESI response when pH value was above 7.4, slightly lower pH in the range of 6.0–7.4 may be more suitable in terms of detection efficiency by NI-ESI. While lower pH under the pH range evaluated, such as 6.0, seems to favor the NI-ESI response to some extent, it may also increase the risk of destroying the speciation for weakly bound complexes. So, pH 7.4, a physiologically pH, is more preferable for the studying of non-covalently bound protein complexes.

3.6. Effect of TRIS acetate concentration on NI-ESI response

Fig. 4c shows the NI-ESI responses of MT-2a in different concentrations of TRIS acetate solution at pH 7.4. As TRIS acetate concentration increases from 1 to 50 mM, a steady increase with decreasing rate of the NI-ESI response was observed. No signal suppression was found even at high concentrations (such as 50 mM) of TRIS acetate buffer. The increasing signal intensity with increasing TRIS acetate concentration is not surprising, since the system would favor the deprotonation of the analytes as described earlier. The slowing down of the increasing trend at high concentrations indicates a lessening of the contributions from this factor at increasingly higher concentrations. The absence of suppression at high concentrations is beneficial for the study of non-covalent macromolecular complexes, which require high ionic strength to maintain complexation in buffer.

4. Conclusions

The mass spectrometric analysis of MT by PI-ESI has progressed dramatically in recent years. However, the analysis of proteins by PI-ESI at neutral pH, a condition necessary for retaining the integrity of the metal binding MT species, suffers the problem of low sensitivity. In this study, a more sensitive method had been developed for the detection of Zn_7-MT-2a by NI-ESI. The system involved the use of TRIS acetate buffer in the neutral pH range. The sensitivity enhancement of the system may be due to the high gas-phase proton affinity, small molecular volume of acetic anion, and the high hydrogen consumption by TRIS in the buffer system. Similar result could be obtained for the cadmium binding MT-2 purchased from Sigma (data not shown). Our work has shown that TRIS acetate is a rather effective buffer system when using NI-ESI-MS detection for native MT. The result also suggests that for experiments that benefit from the use of physiologic pH buffer systems, such as often used in the studying of non-covalent complexes, TRIS acetate buffer may be an attractive system that may offer substantial sensitivity advantages to proteins detection by NI-ESI-MS. Further research includes the long-term stability of the ESI interface while employing this non-volatile buffer.

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