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Journal of Chromatography B, 669 (1995) 27–37

JOURNAL OF CHROMATOGRAPHY B: BIOMEDICAL APPLICATIONS

Comparison of different techniques for the analysis of metallothionein isoforms by capillary electrophoresis

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Abstract

We have investigated free-solution capillary electrophoresis (FSCE) and micellar electrokinetic capillary chromatography (MECC) separations of metallothionein (MT) isoforms conducted in uncoated and surface-modified fused-silica capillaries. At alkaline pH, FSCE rapidly resolves isoforms belonging to the MT-1 and MT-2 charge classes. At acidic pH, additional resolution of MT isoforms is achieved. The use of high-ionic-strength (0.5 *M*) phosphate buffers can result in high peak efficiencies and increased resolution for some MT isoforms. Interior capillary surface coatings such as polyamine and linear polyacrylamide polymers permit separation of MT isoforms with enhanced resolution through their effects on electroosmotic flow (EOF) and protein–wall interactions. Improvements in MT isoform resolution can also be achieved by MECC using 100 m*M* borate buffer pH 8.4 containing 75 m*M* SDS. Deproteinization of tissue cytosol samples with acetonitrile (60–80%) or perchloric acid (7%) produces extracts that can be subjected to direct analysis of MT by FSCE or MECC. We conclude that optimal separation of MT isoforms by capillary electrophoresis (CE) can be achieved with the appropriate combination of different capillaries, buffers and sample preparation techniques.

1. Introduction

Metallothioneins comprise families of closely related proteins with individual members referred to as isoforms. The number of MT isoforms varies among different species. In general, eukaryotic (class I) MTs are characterized by the following properties: (1) low molecular mass (~6000 Da), (2) absence of aromatic amino acids, (3) high content of cysteine (30% of total amino acids), (4) high metal content (7–12 g-atoms of metal/mol) and (5) the presence of two metal-binding domains or clusters [1]. Although a number of functions have been proposed for MT including: heavy metal detoxification, zinc and copper homeostasis, protection against oxidative damage caused by free radicals and as part of the acute phase response to inflammation and stress, a definitive role for MT remains elusive [2]. It is now well established that not only do MTs bind a variety of metals, but many of the metals that bind to the protein also are capable of inducing its synthesis via metal-mediated gene transcription [3]. In addition to metals, other substances such as glucocorticoid hormones, cytokines and endotoxin have been reported to stimulate MT gene transcription [4].

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SSDI 0378-4347(95)00008-9

There have been a limited number of observations concerning the differential regulation of MT gene transcription by metals and other inducing substances. In order to accurately determine if MT genes are coordinately or differentially expressed and to explore the functional significance of individual isoforms, new techniques are required that are capable of resolving and quantifying all of the MT isoforms.

Since the discovery of MT over thirty years ago, there have been a number of techniques developed to isolate and characterize the protein. The most commonly used separation method involves low pressure chromatography consisting of an initial separation using size-exclusion chromatography followed by anion-exchange chromatography which resolves two major charge classes of MTs referred to as MT-1 and MT-2 based on the order of their elution from the column [5]. Since MTs have no aromatic amino acids, detection of the protein following chromatography has been indirect by monitoring bound metals such as zinc and cadmium or the incorporation of radiolabeled precursor molecules such as [^{35}S]cysteine. The requirements for large sample size and the indirect measurements have limited the use of conventional chromatography for accurate quantitative analysis of MT, especially when present at low levels. This situation has been improved somewhat by the use of high-performance liquid chromatography (HPLC) in size-exclusion, anion-exchange and reversed-phase modes [6]. In addition, coupled systems which utilize atomic absorption or inductively coupled plasma emission spectroscopy to detect and quantify metals have enhanced the analytical capability of chromatography-based MT analyses [6–9]. Alternatively, electrochemical detection, either alone or in conjunction with size-exclusion chromatography has been employed to increase the selectivity of detection for MT [10]. The best resolution of MT isoforms to date has been achieved using reversed-phase HPLC (RPHPLC) which is capable of separating isoforms that share a common net charge and differ only in their hydrophobic character [6]. However, RPHPLC analyses which involve the use of expensive columns and organic solvents are relatively lengthy for each sample with times ranging from 30 min to over 1 h to complete. The analytical techniques that provide the highest degree of sensitivity and selectivity are immunoassays and for MT both radioimmunoassays (RIA) and enzyme-linked immunosorbent assays (ELISA) have been developed [11–13]. However, the ability of immunoassays to discriminate among the different MT isoforms depends on the specificity of the antibody used and, at best, only the MT-1 and MT-2 isoform charge classes can be accurately determined [12,14]. Both non-denaturing and denaturing polyacrylamide gel electrophoresis (PAGE) have been utilized to analyze MT isoforms [15,16]. In the case of SDS-PAGE, MTs must first be reduced and alkylated to avoid anomalous behavior caused by excessive binding of SDS to the protein sulfhydryl groups [16]. We have recently been investigating the application of capillary electrophoresis (CE) to the qualitative and quantitative analysis of MT isoforms [17–23]. The overall objective of this work has been to develop a rapid, sensitive and quantitative technique for the analysis of individual MT isoforms isolated from tissue and cultured cell samples. In this study, we compare and contrast different techniques involving the application of CE to the analysis of MT isoforms.

2. Experimental[1]

2.1. Instrumentation

Separations were performed on a P/ACE System (Beckman Instruments, Fullerton, CA, USA) equipped with either a fixed-wavelength UV detector or a photodiode-array detector. In this study, 75, 50 and 20 μm internal diameter (I.D.) fused-silica capillaries of varying lengths were used. Three different types of capillaries

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were tested: (1) untreated, fused-silica (uncoated), (2) treated internally with a polyamine polymer coating (amine) and (3) treated internally with a neutral hydrophilic polyacrylamide coating (neutral). All of the capillaries used were supplied by Beckman Instruments. Each capillary was housed in a cartridge with liquid cooling to maintain capillary temperature at 25°C during the run. Data were collected and processed using System Gold software (Beckman Instruments).

2.2. Materials

Standard Cd,Zn-MTs were purchased from a commercial source (rabbit liver and horse kidney, Sigma, St. Louis, MO, USA). An experimentally prepared rabbit liver Cd,Zn-MT was a gift from Dr. Frederick A. Liberatore (Du Pont Medical Products, N. Billercia, MA, USA). Chicken Zn-MT was prepared from the livers of zinc-injected chickens (four daily injections of 10 mg Zn/kg body weight) by sequential size-exclusion and anion-exchange chromatography steps [5], followed by reversed-phase HPLC [6,8]. All buffers used in this study were prepared from standard reagents in the laboratory or were supplied specifically for use with the individual capillaries (Beckman Instruments).

2.3. Methods

FSCE was performed under the following conditions: Prior to each run the capillaries were treated as follows: (1) the uncoated capillary (50 μm I.D. × 57 cm, 50 cm to detector or 20 μm I.D. × 27 cm, 20 cm to detector) was flushed with deionized water followed by 1.0 *M* NaOH followed by deionized water again and finally with buffer for 2.0 min each, (2) the amine-coated capillary (50 μm I.D. × 57 cm, 50 cm to detector) was flushed for 2.0 min each with wash (1.0 *M* NaOH) and amine regenerator (Beckman Instruments) solutions followed by buffer, (3) the neutral-coated capillary (37 μm I.D. × 57 cm, 50 cm to detector) was washed with buffer for 2.5 min prior to the next run or with 0.1 *M* HCl for 1.0 min followed by buffer for 2.5 min as required to regenerate the capillary. MECC was conducted as described previously [22,23] using an uncoated capillary (75 μm I.D. × 57 cm, 50 cm to detector) and a buffer consisting of 100 m*M* borate and 75 m*M* SDS at pH 8.4. Samples of MT were dissolved in deionized water at a final concentration of 1.0 or 5.0 mg/ml. All samples were loaded hydrodynamically at 3.45 kPa positive pressure for the times specified after which the run was initiated by applying voltage across the capillary at the specified field strength. MT isoforms were routinely detected by monitoring their UV absorbance at 200 nm. When photodiode-array detection was utilized, an absorption spectrum of 190–340 nm (at 1-nm intervals) was collected at 4 points per second for each MT isoform peak detected. The scan obtained at each peak apex was used to characterize the isoform peak.

2.4. Reversed-phase HPLC

Rabbit liver Cd,Zn-MT (1.0 mg/ml in water) was subjected to reversed-phase HPLC as follows: (1) column: Delta-Pak C_{18}, 150 × 3.9 mm I.D. (Waters, Milford, MA USA); (2) buffers: 0.1% TFA (A), 0.1% TFA–60% acetonitrile (B); (3) flow-rate: 1.0 ml/min, (4) two-step elution gradient: 0–30% B in 5 min and 30–40% B in 40 min, (5) MT isoforms were detected by monitoring UV absorbance at 214 nm. Fractions corresponding to each detected peak were collected, lyophilized and redissolved in 100 μl of 0.1% TFA prior to analysis by FSCE.

2.5. Sample preparation

Samples (1 g) of chicken liver were homogenized in 2 ml of deionized water. The homogenate was heated to 100°C for 2 min and centrifuged for 2 min at 10 000 *g*. The supernatant (heat-treated cytosol) was further fractionated by the addition of 1/10th volume of neat (70%) perchloric acid followed by centrifugation or by sequential additions of acetonitrile to produce final concentrations of 60% and 80%. Following centrifugation, the pellet from the treatment with 80% acetonitrile was redissolved in 100 μl of deionized water. The samples in either 7%

perchloric acid or deionized water were analyzed directly by FSCE. When MT standard (chicken Zn-MT) was added, it was added to the homogenate prior to fractionation by solvent or acid. For MECC, extracts of sheep or rat liver were prepared as follows: (1) 1 g of liver was sonicated in 2 ml of 10 m*M* 2-mercaptoethanol, followed by centrifugation, (2) to the resulting supernatant an equal volume of acetonitrile–ethanol (3:1, v/v) was added slowly while vortex-mixing, (3) the sample was centrifuged and to the supernatant additional acetonitrile–ethanol was added to 80% and the sample centrifuged, (4) the pellet was redissolved in 50 μl of deionized water and an aliquot was analyzed by MECC.

3. Results and discussion

Fig. 1 depicts the rapid separation of Cd,Zn-MTs from rabbit liver (Fig. 1A) and horse kidney (Fig. 1B) by FSCE. When an alkaline borate buffer (100 m*M* borate, pH 8.4) is used in conjunction with an uncoated fused-silica capillary, it is possible to separate the two major charge classes of MTs (MT-1 and MT-2). This type of separation is analogous to that obtained by subjecting MTs to anion-exchange chromatography [5], except that with FSCE the analysis time is much shorter. However, it is well known from studies employing reversed-phase HPLC that both of these MTs exhibit additional heterogeneity [24]. Both rabbit and horse MTs have been reported to be comprised of up to six individual isoforms, although not all of which are present in equal amounts [24–26]. Therefore, we investigated the use of alternative buffers and conditions in an attempt to separate as many of the individual isoforms as possible. We reported previously that phosphate buffers offered two important advantages when used in conjunction with fused-silica capillaries for the separation of MT isoforms [19]. First, the interaction of phosphate with the capillary wall resulted in a reduction of electroosmotic flow (EOF) and a subsequent improvement in the resolution of MT isoforms. Second, unlike Tris or other organic-based buffers, phosphate does not absorb significantly in the low UV region and therefore, permits more sensitive detection of MT isoforms at 200 nm. This is an important consideration in light of the fact that all MTs characteristically lack aromatic amino acids and thus exhibit maximum absorbance at about 200 nm due to the contribution of the peptide bonds. Thus, in order to achieve the highest sensitivity in the analysis of MTs subjected to CE, detection at this wavelength is recommended. Fig. 2 depicts the separation of rabbit liver and horse kidney MTs using 50 m*M* sodium phosphate buffer at pH 7.0 (Fig. 2A,C) and pH 2.0 (Fig. 2B,D). At pH 7.0, the phosphate buffer provides additional resolution of MT isoform peaks compared to that achieved with the alkaline borate buffer (Fig. 1). Further resolution is achieved when the pH of the phosphate buffer is lowered to 2.0. It has been previously reported that, at this pH, the bound zinc and cadmium would completely dissociate from the protein leaving the metal-free proteins or apothioneins [1]. This is supported by the data shown in Fig. 3 which depicts peak UV absorption spectra obtained for rabbit liver

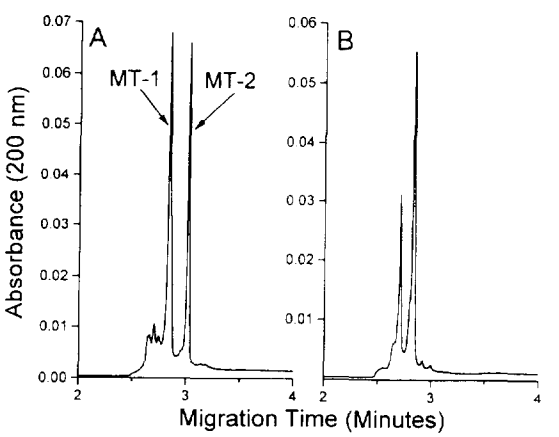

Fig. 1. Electropherograms of rabbit liver (A) and horse kidney (B) Cd,Zn-MTs. MTs were dissolved at a concentration of 1.0 mg/ml in deionized water and loaded onto the capillary by pressure injection for 10 s. A 50 μm I.D. × 57 cm (50 cm to detector) fused-silica, uncoated capillary was used. The running buffer was 0.1 *M* borate pH 8.4 and the running voltage was 30 kV, normal polarity (outlet negative). The MT-1 and MT-2 peaks are so indicated for rabbit liver MT (A).

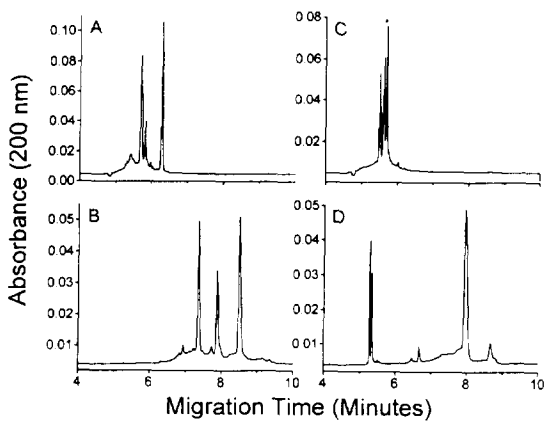

Fig. 2. Electropherograms of rabbit liver (A,B) and horse kidney (C,D) Cd,Zn-MTs. The separation conditions were the same as those described in the legend to Fig. 1 except that two running buffers were used: 50 m*M* sodium phosphate pH 7.0 (A,C) and 50 m*M* sodium phosphate pH 2.0 (B,D).

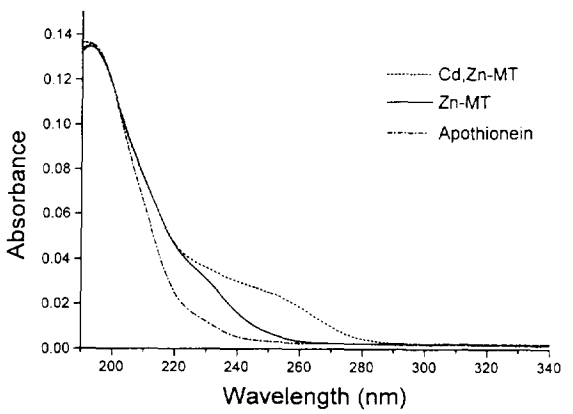

Fig. 3. Normalized UV absorption spectra for MT isoform peaks separated by FSCE. Each line represents a scan between 190 and 340 nm at 1-nm intervals with the data collected at 4 points per second. The scans depicted represent those obtained at the apex of each peak detected. The Cd,Zn-MT represents the peak migrating last in Fig. 2A, the Zn-MT represents chicken MT (electropherogram not shown). Both samples were separated as described in the legend to Fig. 1 except that 50 m*M* sodium phosphate running buffer at pH 7.0 was used. The apothionein data was obtained from the peak migrating last in Fig. 2B which was separated using 50 m*M* sodium phosphate running buffer at pH 2.0.

Cd,Zn-MT-2a (peak migrating last in Fig. 2A), and chicken Zn-MT (electropherogram data not shown) both separated with 50 m*M* sodium phosphate buffer at pH 7.0 and rabbit liver Cd,Zn-MT-2a run at pH 2.0 (peak migrating last in Fig. 2B). The metal–thiolate bonds in MTs exhibit distinct absorption spectra with the Cd-S low-energy charge-transfer transition band at 250 nm and the Zn-S at 231 nm [5,27]. It is also clear from Fig. 3 that the MT peak from the run employing sodium phosphate buffer at pH 2.0 exhibits an absorption spectrum characteristic of apothioneins [27]. We had previously speculated that CE conducted at acidic pH resulted in the separation of apo- rather than holo-(metallo)-thioneins [19], although we did not have direct evidence to support that assumption. Now, based on these absorption spectra obtained, we conclude that FSCE conducted at pH 2.0 results in the separation of apothioneins with their metals having dissociated at some point during the run. Moreover, at neutral or alkaline pH, metals remain associated with the resolved isoform peaks as is evidenced by the distinctive absorption spectra for the Zn-MT and the Cd,Zn-MT. It is also important to note that MT-bound copper under acidic (pH 2) conditions probably would not dissociate since the Cu–S bond remains intact at this pH [1]. Thus, the introduction of commercially available photodiode-array detectors for CE instruments has now made it possible to derive useful information from absorption spectra of the MT isoform peaks resolved that is indicative of the type and status of bound metals.

The effect of ionic strength of a sodium phosphate buffer at pH 2.0 is depicted in Fig. 4. Increasing the ionic strength from 10 m*M* to 150 m*M* results in a reduction of EOF, peak sharpening and enhanced resolution of the isoform peaks for rabbit liver Cd,Zn-MT. Throughout the range of ionic strengths tested, three major peaks separate. We have investigated the effects of using ionic strengths up to 0.5 *M* sodium phosphate pH 2.0, however, in order to use such high-ionic-strength buffers, it is necessary to work with narrower bore capillaries (20 μm I.D.) and shorter lengths (27 cm). Under these

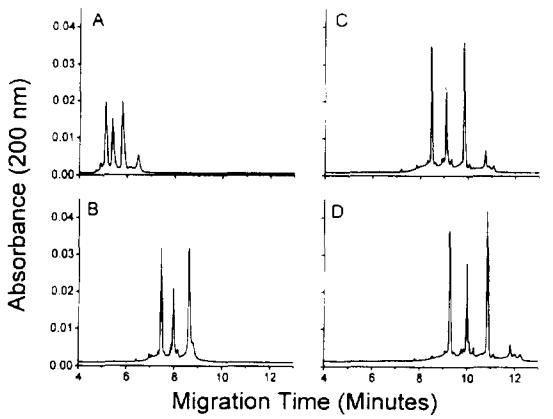

Fig. 4. Electropherograms of rabbit liver Cd,Zn-MT separated according to the conditions described in Fig. 1 except that a phosphate running buffer pH 2.0 was used at the following concentrations: 10 m*M* (A), 50 m*M* (B), 100 m*M* (C), and 150 m*M* (D).

conditions some additional resolution is noted for the fastest migrating peak for rabbit liver Cd,Zn-MT (Fig. 5A). Horse kidney Cd,Zn-MT shows a similar degree of isoform resolution compared to phosphate buffers of lower ionic strength (Fig. 2D). However, in both cases, equal or better resolution of the MT isoforms is

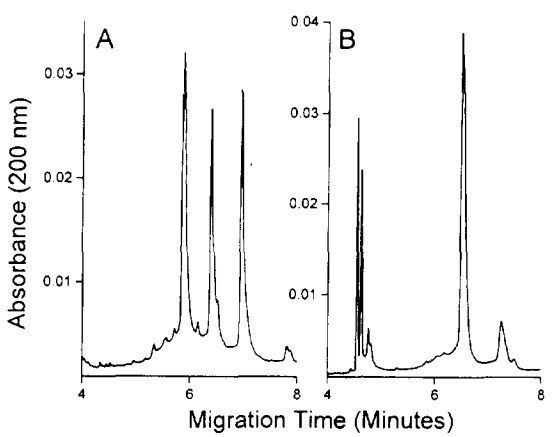

Fig. 5. Electropherograms of rabbit liver (A) and horse kidney (B) Cd,Zn-MTs. MTs were dissolved at a concentration of 5.0 mg/ml in deionized water and loaded into the capillary by pressure injection for 10 s. A 20 μm I.D. × 27 cm (20 cm to detector) fused-silica, uncoated capillary was used. The running buffer was 0.5 *M* sodium phosphate pH 2.0 and the running voltage was 15 kV, normal polarity (outlet negative).

achieved in a shorter time as compared to separations using wider-bore and longer-length capillaries with lower ionic strength phosphate buffers (Figs. 2 and 4). The advantages of using very high (i.e., 0.5 *M*)-ionic-strength phosphate buffers include: (1) additional improvements in isoform resolution, (2) the ability to shorten the run time while preserving MT isoform peak resolution by utilizing shorter capillaries and (3) the ability to directly analyze MT-containing samples that contain high endogenous levels of salts such as fractions from ion-exchange chromatography. One potential disadvantage of using high-ionic-strength phosphate buffers is that they have a high conductivity and thus rigorous control of capillary temperature is essential to minimize or prevent the adverse effects from Joule heating. Depending on the type and capabilities of CE instrumentation available, this may limit the utility of these buffers for MT isoform separations.

In an attempt to verify the identity of the peaks resolved by FSCE using the acidic phosphate buffer, we first subjected a sample of rabbit liver Cd,Zn-MT to reversed-phase HPLC (RPHPLC) using 0.1% trifluoroacetic acid buffer with a two-step linear gradient of acetonitrile. The net result is depicted in Fig. 6. Under these conditions five major peaks were resolved by RPHPLC (Fig. 6A), four of which corresponded to specific MT isoforms as described previously by Wan et al. [25,26]. We collected each peak resolved by RPHPLC separately and ran them on FSCE in order to determine their order of migration. Insufficient material was available to assign an identity to each of the lesser abundant peaks, but it is clear that the earliest and last migrating peaks detected on FSCE (Fig. 6B) are comprised of MT-2 isoform subtypes, whereas the intermediate peak corresponds to MT-1a.

Since low-pH buffers appeared to give maximum resolution in uncoated fused-silica capillaries, we next decided to investigate modifications to the internal wall of the capillary used to perform the separations. Fig. 7 depicts the separation of rabbit liver (A) and horse kidney (B) Cd,Zn-MTs in a capillary coated with a polyamine polymer that reverses EOF by imparting a

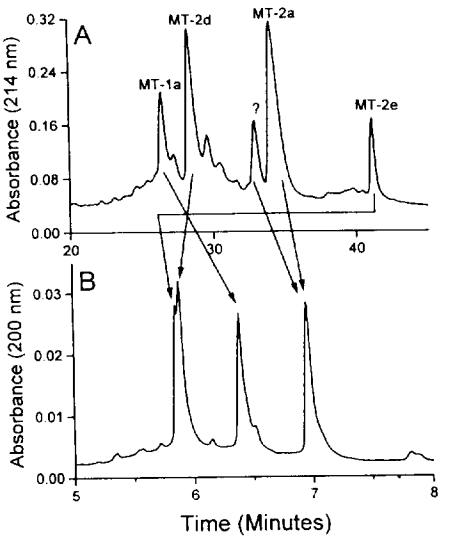

Fig. 6. Separation of rabbit liver Cd,Zn-MT by RPHPLC (A) and FSCE (B). The conditions for RPHPLC are described in the text and the conditions for FSCE were those described in the legend to Fig. 5. Individual MT isoform peaks are designated with the number referring to the charge class and the letters designating individual subtypes. Fractions corresponding to each peak separated by RPHPLC were collected, lyophilized and redissolved in 0.1% TFA. The assignment of peaks separated by RPHPLC to peaks resolved by FSCE (designated with arrows) was determined by subjecting samples of each peak collected to FSCE.

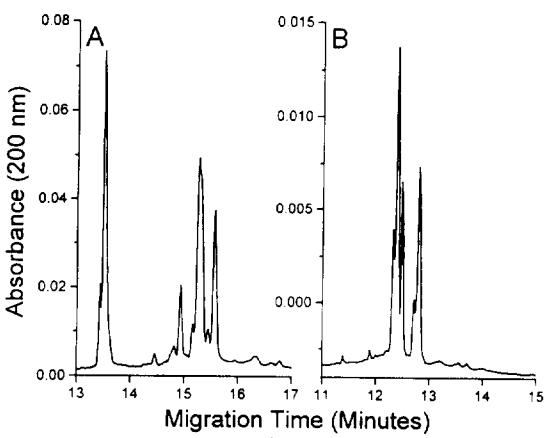

Fig. 7. Electropherograms of rabbit liver (A) and horse kidney (B) Cd,Zn-MTs. MTs were dissolved at a concentration of 1.0 mg/ml in deionized water and loaded onto the capillary by pressure injection for 10 s. A 50 μm I.D. × 57 cm (50 cm to detector) fused-silica, polyamine-coated capillary was used. The running buffer was 0.1 *M* sodium phosphate adjusted to pH 7.0 and the running voltage was 20 kV, reversed polarity (outlet positive).

positive charge to the capillary wall. The use of 0.1 *M* sodium phosphate buffer pH 7.0 gave the best results of all buffers tested and additional resolution was obtained compared to an uncoated capillary (see Figs. 2A,C). Because of the surface charge reversal, reversed polarity is used to conduct FSCE and as a result the order of migration is reversed from that observed at this pH in an uncoated capillary (see Fig. 2A,C). Since under these conditions MT isoforms retain a net negative charge, their electrophoretic mobilities (μ_e) would be aligned with EOF (i.e. toward the anode) compared to an uncoated fused-silica capillary at this pH run at normal polarity where EOF and μ_e would be aligned in opposite directions. This balance between EOF and μ_e could account for the observed improvements in resolution of isoform peaks. In general, we have found that the use of this capillary produced superior resolution of MT isoforms for a number of different MTs surveyed [20,21].

Another type of coated-capillary which was evaluated with respect to the resolution of MT isoforms was one in which the internal surface was treated with a neutral, hydrophilic, polyacrylamide coating. In this case, EOF is greatly suppressed as is the interaction of proteins with the wall. One unique advantage of this particular capillary is that with an appropriate choice of buffer, FSCE can be conducted using either normal or reversed polarity. This situation allows for the exploitation of differences in the net positive or net negative charge on MT isoforms which determine their migration toward the anode or cathode. An example of this is depicted in Fig. 8 in which the same sample of rabbit liver Cd,Zn-MT was separated using 50 m*M* sodium phosphate buffer at pH 7.0 and reversed polarity (A) or at pH 2.0 with normal polarity (B). Clearly the degree of isoform separation varies between the two, but the separation at pH 7.0 shows further resolution beyond that achieved using uncoated (see Fig. 2A) or polyamine-coated (see Fig. 7A) capillaries.

Another approach we have investigated to resolve MT isoforms involves MECC [22,23]. The conditions chosen were to add SDS (75 m*M*) to a 100 m*M* borate buffer at pH 8.4 used

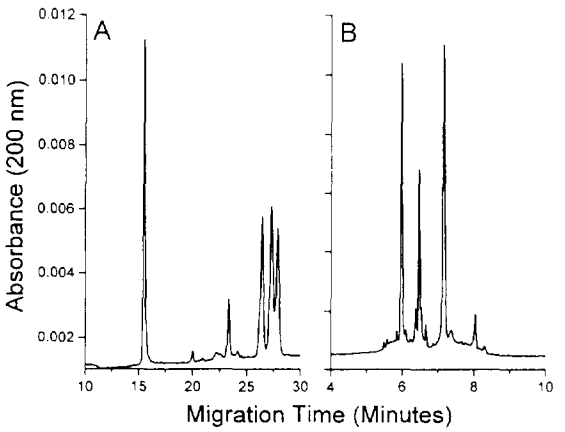

Fig. 8. Electropherogram of rabbit liver Cd,Zn-MT. MT was dissolved at a concentration of 1.0 mg/ml in deionized water and loaded onto the capillary by pressure injection for 3 s. A 50 μm I.D. × 37 cm (30 cm to detector) fused-silica, neutral polyacrylamide-coated capillary was used. Two running buffers and different polarities were used: (A) 50 m*M* sodium phosphate adjusted to pH 7.0 with a running voltage of 18.5 kV, reversed polarity (outlet is positive) and (B) 50 m*M* sodium phosphate pH 2.0 with a running voltage of 18.5 kV, normal polarity (outlet is negative).

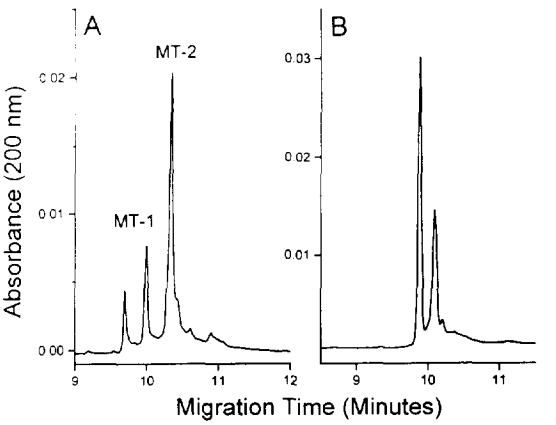

Fig. 9. Electropherograms of rabbit liver (A) and horse kidney (B) MTs separated by MECC. MTs were dissolved at a concentration of 1.0 mg/ml in deionized water and loaded into the capillary by pressure injection for 1 s. A 75 μm I.D. × 57 cm (50 cm to detector) uncoated fused-silica capillary was used. The running buffer was 0.1 *M* borate–75 m*M* SDS (pH 8.4) and the running voltage was 10 kV, normal polarity (outlet negative). The MT-1 and MT-2 peaks are indicated for rabbit liver MT (A).

in the separation. Compared to FSCE in borate buffer alone at this pH (see Fig. 1), additional resolution of MT isoforms was achieved by the use of MECC conditions for the separation of rabbit liver Cd,Zn-MT (Fig. 9). The resolution for horse kidney MT was not significantly affected, with two major isoform peaks being separated in both FSCE and MECC modes. One distinct advantage of the MECC mode of separation is in the analysis of complex sample matrices which typically exhibit many other peaks besides those corresponding to the MT isoforms. For example, cytosol extracts prepared from rat and sheep liver using an acetonitrile–ethanol (3:1, v/v) deproteinization procedure [23] were analyzed for MT isoforms using MECC (Fig. 10). Although complete resolution of individual MT isoforms was not achieved in both cases, this mode of CE does provide sufficient resolution to quantitatively determine the levels of individual isoform peaks. The degree of resolution and thus the utility of MECC may depend on a number of different variables such as species, tissue type and degree of MT isoform heterogeneity as well as prior sample preparation. This is indicated by a comparison of rat vs. sheep samples where the MT isoforms from sheep liver show a higher degree of resolution than for rat liver in which the MT-2 isoform peak migrates in close proximity to an abundant uncharacterized component (Fig. 10). It is pos-

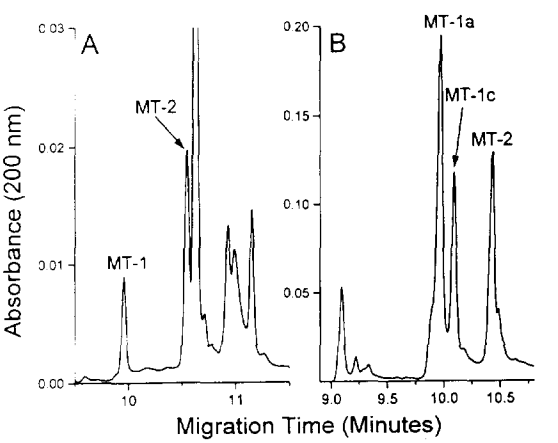

Fig. 10. Separation of extracts from rat (A) and sheep (B) liver by MECC. Extracts were prepared by an acetonitrile–ethanol extraction procedure described in the text. The separation conditions were the same as those described in the legend to Fig. 9. The MT-1 and MT-2 isoform peaks are indicated.

sible to optimize the MECC separation of specific MTs by altering the pH, ionic strength and SDS concentration of the buffer used which can result in better resolution of the individual isoform peaks [23].

We have also attempted to analyze cytosol extracts by means of FSCE. To do this, we prepared extracts of chicken liver by a two-step acetonitrile deproteinization procedure [22] followed by separation of the extract in an uncoated fused-silica capillary (20 μm I.D. × 27 cm) using 0.5 *M* sodium phosphate buffer at pH 2.0 (Fig. 11). Because uninduced or control tissue does not contain much detectable MT, a method of standard additions was tried in order to estimate the amount of endogenous MT present. Since chickens possess only a single predominant form of MT [28], this type of analysis was simplified considerably. Fig. 11 depicts the results from a preliminary analysis of control, control plus 100 μg or 200 μg of added chicken Zn-MT standard/g of liver. It is clear

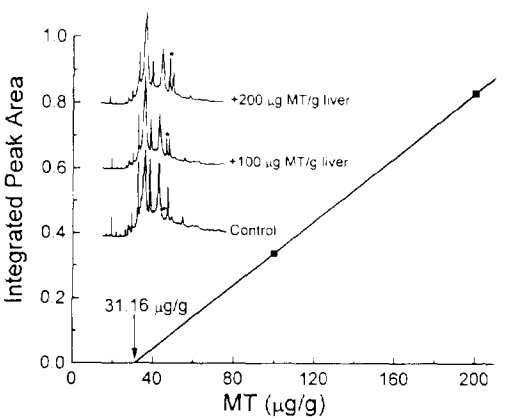

Fig. 11. Quantitative determination of MT content in chicken liver by a method of standard additions. An extract of control chicken liver was prepared using an acetonitrile deproteinization procedure described in the text. The extract (control) or extracts from samples to which 100 or 200 μg/g liver of purified chicken Zn-MT was added were subjected to FSCE as described in the legend to Fig. 5. A portion of each electropherogram is shown in the inset with the MT peak designated (*). Integrated peak areas for MT were calculated and plotted vs. the quantity of added MT. The estimate of MT content (31.16 μg/g liver) for control tissue was derived from the extrapolation of the line through zero area.

from the electropherograms that only a single peak responded to the addition of MT standard. If the integrated peak areas are plotted and the regression line extrapolated to zero area, an estimate of 31.16 μg MT/g of liver is obtained which compares favorably with published values of control chicken liver MT [29]. We have previously shown for FSCE conducted using both uncoated and amine-coated capillaries that a linear relationship exists between integrated peak area and the concentration of MT in the sample injected onto the capillary [17,18,21]. Thus, the combination of an appropriate sample pre-treatment combined with the resolving power of FSCE suggests a basis for establishing a quantitative assay for tissue levels of MT. However, the limit of detection (LOD) for a 20 μm I.D. capillary is considerably higher than the 1–5 μg/ml estimated for 75 or 50 μm I.D. capillaries [17,18,22]. An analysis of control and zinc-induced chicken liver cytosol extracts for MT was also reported using the amine-coated capillary and the level of MT in control samples was found to be too low to quantify accurately [19]. Thus, detection sensitivity continues to be a major obstacle to analysis of tissue samples for MT, especially those tissues which do not contain high levels to begin with. Moreover, it is likely that further trials using alternative buffers and capillaries will produce conditions better suited to the analysis of MT isoforms contained in crude tissue extracts.

Fig. 12 depicts the separation of a liver extract from zinc-injected chickens that was subjected to deproteinization by perchloric acid (final concentration 7%). The MT peak was detected (Fig. 12A) and verified by the addition of 100 μg of additional chicken Zn-MT standard/g of liver (Fig. 12B) which caused only the MT peak to increase. This work demonstrated the feasibility of using an acidic aqueous extraction procedure as an alternative to organic solvents to remove extraneous protein. Since MT is known to be tolerant of acidic conditions including perchloric acid [1], it is logical to attempt to exploit this characteristic in the preparation of samples for CE analysis. The use of acidic extraction conditions may also be advantageous in preventing

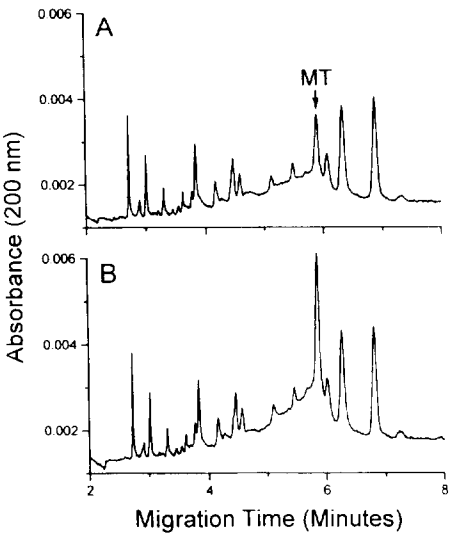

Fig. 12. Separation of a perchloric acid extract of liver from a zinc-injected chicken (A). The extract was subjected to FSCE as described in the legend to Fig. 5. An additional 100 μg/g liver of purified chicken Zn-MT was added to verify the peak corresponding to MT (B).

potential oxidation of the abundant sulfhydryl groups in MTs which are known to be prone to oxidation and polymerization during tissue homogenization [30]. This technique has been successfully applied to the extraction and purification by HPLC of the calcium-binding protein, calbindin (9 kDa) from rat intestinal tissue [31]. However, more work is required to determine the stability of different MTs in the presence of 7% perchloric acid, especially the long-term stability as well as the overall effectiveness of the acidic extraction method. It may also be possible to combine acid extraction with either heat and or solvent deproteinization methods to produce a cleaner extract better suited to direct analysis of MT isoforms by FSCE. We are continuing to explore these and other possibilities with the goal of improving sample pretreatment procedures prior to the analysis of MTs by CE.

4. Conclusions

In this study we have compared and contrasted various techniques and procedures for the separation of MT isoforms by CE from previously purified samples and tissue extracts. The choice of capillary, buffer and sample preparation conditions all have an important impact on the analysis. We conclude, for the analysis of MTs which exhibit little heterogeneity beyond the occurrence of the two major charge classes that FSCE separations using an uncoated capillary and an alkaline buffer system will provide a rapid analysis with sufficient resolution to form the basis for a quantitative analytical technique. For those MTs that exhibit significant microheterogeneity with respect to the occurrence of MT isoforms or complex sample matrices containing MT isoforms, additional resolution can be achieved in several ways including: (1) use of an acidic buffer, (2) use of specific coated capillaries, (3) employing MECC separation conditions or (4) pre-treatment of the sample prior to CE. Photodiode-array detection offers useful information about the metal complement of the individual MT isoforms because of the unique absorption spectra characteristic of metal–thiolate bonds. Future developments in sample deproteinization and detection methodology are needed to extend the sensitivity and the selectivity necessary to make CE a viable method for MT isoform analysis in a wider variety of samples.

Acknowledgements

The assistance of Julie Ficke and Dr. Paul Shieh at Beckman Instruments (Fullerton, CA, USA) in providing the amine- and neutral-coated capillaries used in this study and for advice concerning their use is gratefully acknowledged. The authors also wish to acknowledge Professor Ian Bremner and Dr. John Arthur at The Rowett Research Institute for their critical evaluation of this manuscript. A portion of this work was funded by the Scottish Office Agriculture and Fisheries Department, UK.

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