Chapter 28

Modern Bioanalysis of Proteins by Electrophoretic Techniques

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Abstract

In 1957, protein rich in cysteine able to bind cadmium was isolated from horse kidney and named as metallothionein according to its structural properties. Further, this protein and metallothionein-like proteins have been found in tissues of other animal species, yeasts, fungi and plants. MT is as a potential cancer marker in the focus of interest, and its properties, functions, and behavior under various conditions are intensively studied. Our protocol describes separation of two major mammalian isoforms of MT (MT-1 and MT-2) using capillary electrophoresis (CE) coupled with UV detector. This protocol enables separation of MT isoforms and studying of their basic behavior as well as their quantification with detection limit in units of ng per μL. Sodium borate buffer (20 mM, pH 9.5) was optimized as a background electrolyte, and the separation was carried out in fused silica capillary with internal diameter of 75 μm and electric field intensity of 350 V/cm. Optimal detection wavelength was 254 nm.

Key words Metallomics, Gel electrophoresis, Capillary electrophoresis, Chip electrophoresis, Electrochemistry

1 Introduction

1.1 Metallothioneins

Metallothioneins (MTs) were discovered by Margoshes and Vallee in 1957 as newly identified proteins isolated from a horse renal cortex tissue [1]. These proteins occur in whole animal kingdom with high degree of homology. Similar proteins are expressed by bacteria, fungi, and even plants. MTs are low-molecular-mass (from 2 to 16 kDa) proteins with unique abundance of cysteine residues (more than 30 % from all amino acids) (Fig. 1). The main function of MTs in organism is a metal ion transport, maintenance of the oxidative-reducing conditions, and regulation of gene expression. MT regulates free radical level also indirectly by binding of metal ions which are potential radical producers, e.g., Cu [2, 3].

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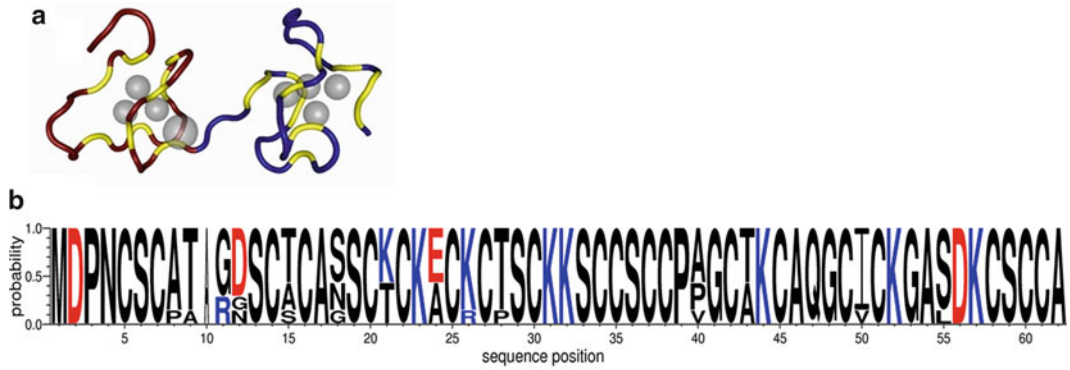

Fig. 1 (**a**) Schematic structure of MT. (**b**) Representative logo—the probability of certain amino acid to occur in certain position (the probability increases with the size of the letter). The change fraction was expressed in percentage of the ratio of each amino acid occurrence on certain position. Acidic and basic amino acids were color coded: neutral—*black*, acidic—*red*, basic—*blue*. Logo was created in professional application WebLogo3

Due to the importance of MT, understanding of its structure, function, and behavior is essential, and therefore effective, sensitive, and reliable analytical method is required. However the complexity of real biological samples requires employment of not only sample pretreatment methods involving extraction and purification but also separation techniques prior to the selected detection. Chromatographic and electromigration methods are well-established commonly used methods enabling elimination of potentially interfering compounds and improving significantly the determination of the target analyte [4–6].

1.2 Gel Electrophoresis

Gel electrophoresis (GE) is a routine method used in biochemical laboratories for identification of bioanalytes such as proteins and/or nucleic acids. Due to MT's structural and chemical properties as low molecular weight, heavy metal content, and high-abundant thiol groups, the protocols commonly used for protein electrophoresis may bring poor results [5, 7]. Generally, isolation of metalloproteins with non-covalently bound metal requires a mild, non-denaturing conditions. Treatment of metalloprotein with denaturing sodium dodecyl sulfate (SDS) gel electrophoresis will result in a loss of metal [8]. Due to low molecular mass of MTs and their easy reoxidation during electrophoretic run, GE analysis may be challenging, and gels with acrylamide concentration approx. 15–17.5 % or gradient gel electrophoresis have to be used. SDS-polyacrylamide gel electrophoresis (PAGE) and electrochemical methods were used for monitoring arsenic influence on MT levels of clams (*Corbicula fluminea*) [9]. In the paper focusing on protein with affinity for Zn and Cd in HeLa cells separation was achieved by metal-chelating column chromatography for protein separation according to their affinity for these metals and bond strength [10]. Moreover, SDS-PAGE combined with specific

fluorescence detection has been showed as a very sensitive tool for determination of MTs in biological samples of various types [11–13].

Tricine–SDS-PAGE is commonly used to separate proteins in the mass range 1–100 kDa. Tricine (*N*-(2-hydroxy-1,1-bis(hydroxymethyl)ethyl)glycine), used as the trailing ion, allows a resolution of small proteins at lower acrylamide concentrations than in glycine–SDS-PAGE systems. It is the preferred electrophoretic system for the resolution of proteins smaller than 30 kDa [14–16]. Isoelectric focusing (IEF), also known as electrofocusing, is a technique for separation of different molecules by their electric charge differences. IEF was also used to separate and to detect metallothioneins and metalloproteins from human liver cytosol [17, 18], mammalian tissues [19], and urine [20] and to study metallothionein-like substance from cyanobacterium *Synechocystis* [21]. IEF is the first step in two-dimensional gel electrophoresis, in which proteins are first separated by their p*I* and then further separated by molecular weight through SDS-PAGE.

1.3 Capillary Electrophoresis

Since the introduction of capillary electrophoresis (CE) by Jorgenson and Lukacs in 1981 [22], the technique has rapidly developed into a versatile analytical tool. As the MT is concerned, separation of its isoforms and metal affinity studies are the main areas of CE application. Already in 1998, the strategies for the qualitative and quantitative analysis of metallothionein isoforms by capillary electrophoresis were summarized by Beattie [23]. Several researchers, such as Richards and Beattie [24–35], Minami and coworkers [36–46], Virtanen [47–52], Wilhelmsen [53–55], and others [4, 56, 57], studied systematically CE of MT.

Three main factors such as electrolyte, capillary, and detection method have to be optimized in order to reach required resolution and sensitivity within a reasonable time scale. Even though the majority of CE experiments are carried out in aqueous solutions such as borate, phosphate, acetate, and others, also nonaqueous capillary electrophoresis methods have been developed [58–60]. Already in 1993, Beattie et al. [26] introduced a CE method for MT isoform separation employing 10 mM Tris–HCl buffer with pH 9.1. Also, separation of MT isoforms from horse, rabbit, and rat at low pH was demonstrated [55]. In 1996, Virtanen et al. presented a systematic study of the impact of buffer composition, concentration, and pH as well as temperature and voltage on separation of MT isoforms [51]. Later, the same author introduced CE method using Tris–tricine buffer containing methanol as a BGE [49]. Tricine buffer as well as Tris–borate buffer were employed in the following studies by Virtanen et al. [49, 50]. For MT determination numerous additives have been tested for separation improvement. Electrolyte modifiers such as methanol, ethanol, propanol, acetonitrile, and acetone were tested by Virtanen et al. in 1998 [52]. Modification of BGE by cyclodextrines for MT isoform analysis

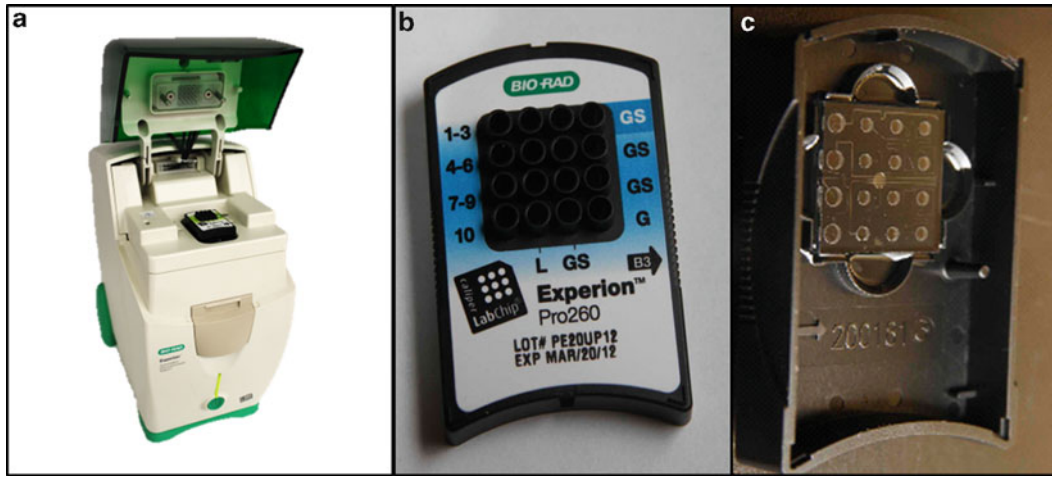

Fig. 2 Photograph of commercial chip CE system. (**a**) The instrument; (**b**, top view) the chip for protein analysis; (**c**, bottom view) the chip for protein analysis

was presented by Wilhelmsen et al. in 2004 [53]. Depending on the concentration of the electrolyte modifier, principally different separation mode called micellar electrokinetic chromatography (MEKC) can be introduced. Systematic optimization of MEKC method in terms of capillary length, pH, buffer, and SDS concentration used as a buffer modifier was carried out for MT determination [25].

Even though numerous CE analyses of MTs have been performed in uncoated silica capillaries [40, 51], several articles describing utilization of surface-modified capillaries to avoid or diminish the undesirable interactions of the analyte with the capillary wall have been published. One of the most widely used surface coatings for MT separations is a linear polyacrylamide [28, 37–41, 43–45]. Polyamine-based surface modifications were also utilized for MT separation [28, 32]. Several main modes of detection in CE are photometric (absorbance), fluorimetric [61–63], and mass spectrometry detection [64–74].

1.4 Chip-Based CE

Miniaturized integrated analytical systems have matured greatly since the initial lab-on-a-chip concept was proposed [75–78]. One of them was used for MT analysis by Krizkova et al. [62, 63] (Fig. 2). In the first paper, chip-based CE was used for quantitative study of MT oxidation by H_2O_2 resulting in significant decrease in peak heights and shift of peak positions to higher molecular mass, corresponding with the time of oxidation. Moreover, it was observed that the proportion of high molecular forms of MT was markedly increased.

The oxidative changes were successfully reversed by using reducing agent prior to electrophoresis. In the second study, the

chip-CE with fluorescence detection was used to determine structural changes of MT with increasing concentration of zinc(II) ions and under various redox conditions. Formation of MT aggregates with increasing zinc concentration was observed by spectrophotometry, chip-CE, and SDS-PAGE. It was found that reduced MT forms aggregate more readily compared to oxidized MT. Using the chip-CE allowed relative quantification of MT aggregation as a decrease in the area of the signal corresponding to the monomer form of MT [63].

2 Materials

Prepare all solutions using ultrapure water (ACS quality) and store at 4 °C (unless stated otherwise). Follow the waste disposal regulation when disposing waste materials.

2.1 Fast Protein Liquid Chromatography

1. 10 mM Tris–HCl buffer for liver sample preparation: Dissolve 1.21 g of Trizma base in 1 L of ultrapure water. Adjust the pH to 8.6 by concentrated HCl.
2. Fast protein liquid chromatography (FPLC) mobile phase: To prepare 150 mM NaCl solution in Tris–HCl buffer dissolve 8.7 g of NaCl in 1 L of 10 mM Tris–HCl (pH 8.6).
3. Perform FPLC using a Biologic DuoFlow system (Biorad, USA), consisting of two chromatographic pumps for the application of elution buffers, a gel-filtration column (HiLoad 26/60, GE Healthcare, Uppsala, Sweden), an injection valve with 2 mL sample loop, an UV–VIS detector, and an automated fraction collector.
    - Use standard glass and plastic consumables for FPLC.

2.2 Gel Electrophoresis

2.2.1 Solutions for Gel Casting

1. Acrylamide and bisacrylamide: Prepare a solution of acrylamide 30 % (*w/v*) and bisacrylamide 1 % (*w/v*) in deionized water.
2. Tris buffer: 3 M Tris pH 8.45; 0.3 % (*w/v*) SDS in deionized water.
3. Glycerol solution: 50 % glycerol (*v/v*) in deionized water.
4. Ammonium persulfate (APS) solution: Weigh out 0.1 g of APS, and add 1 mL of deionized water. Prepare fresh weekly.
5. Tetramethylethylenediamine (TEMED): Transfer 1 mL of TEMED in an Eppendorf tube.

2.2.2 Running Buffer

Prepare running buffer by mixing of 100 mL of 10× Tris/Tricine/SDS Buffer (BioRad, USA) with deionized water. Composition of 1× buffer indicated by manufacturer: 100 mM Tris, 100 mM tricine, 0.1 % (*w/v*) SDS, pH 8.3.

2.2.3 Sample Buffer

Prepare 200 mM Tris–HCl, pH 6.8, 2 % (*w/v*) SDS, 40 % (*v/v*) glycerol, 0.04 % (*w/v*) Coomassie G-250 in deionized water. Immediately mix in ratio 19:1 with β-mercaptoethanol. After mixing with β-mercaptoethanol store for max 24 h.

2.2.4 Solutions for Zinc Staining

Reversible staining kit for polyacrylamide gels includes 10× zinc stain solutions A and B and 10× zinc destain (Bio-Rad, USA).

1. 1× solution A: 0.2 M imidazole and 0.1 % (*w/v*) SDS.
2. 1× solution B: 0.3 M $ZnSO_4$.
3. Destaining solution: 25 mM Tris, 192 mM glycine, pH 8.3.

2.2.5 Solutions for Silver Staining

To prepare 500 mL of the staining solutions, weigh out or pipette the following chemicals in a volumetric flasks and fill in with deionized water.

Solution 1: 5.7 mL of acetic acid, 32 mL of methanol, and 0.5 mL of 37 % (*v/v*) formaldehyde.

Solution 2: 250 mL of methanol.

Solution 3: 0.1 g of sodium thiosulfate.

Solution 4: 1 g $AgNO_3$, 0.38 mL of 37 % (*v/v*) formaldehyde.

Solution 5: 30 g of sodium carbonate, 2 mg of sodium thiosulfate, and 0.25 mL of 37 % (*v/v*) formaldehyde.

Solution 6: 32 mL of methanol and 5.7 mL of acetic acid.

2.2.6 Gel Documentation

After reversible zinc staining the gels can be scanned or photographed against dark background or on UV transilluminator (*see* **Note 1**). After silver staining the gels can be scanned or photographed on a gel-imaging system or an office scanner.

- Use standard glass and plastic consumables for gel electrophoresis.

2.3 Capillary Electrophoresis

1. Background electrolyte: Prepare 20 mM sodium borate buffer at pH 9.5 by dissolving 0.1905 g of sodium tetraborate in 100 mL of ultrapure water (ACS), and adjust the pH with 1 M NaOH.
2. Urea solution: Prepare 14 M urea solution by dissolving of 8.4 g of urea in 10 mL of ultrapure water. Sonicate until it is dissolved.
3. Standard MT solutions: Prepare stock solutions of rabbit liver MT isoforms (MT-1, MT-2) in ACS-quality water (1 mg/mL) and store in the dark at −20 °C. Working standard solutions (0.2 mg/mL) were prepared daily by dilution of the stock solutions with ACS water. Dissolve the MT obtained by FPLC isolation in ACS water to get the concentration of 0.2 mg/mL.

- Use standard glass and plastic consumables for capillary electrophoresis.

2.4 Chip CE

1. Experion Pro260 analysis kit: Modifications of the manufacturer's instructions: (1) Dilute the sample buffer in ratio 1:1 with water. (2) Use only 10 μL of Pro260 stain instead of 20 μL when preparing the gel stain solution.
   - Use standard glass and plastic consumables for chip electrophoresis.

3 Methods

Carry out all procedures at room temperature unless stated otherwise.

3.1 Preparation of Sample for MT Isolation

Homogenize 2 g of defrosted rabbit liver (Ultra-turrax T8, Schoeller instruments, DE) in 8 mL of 10 mM Tris–HCl buffer (pH 8.6).

Vortex it (Vortex Genie), and centrifuge (Universal 320, HettichZentrifugen) it at 2,370 × *g* for 30 min at 4 °C. Take the supernatant and centrifuge it again (Eppendorf centrifuge 5417R) in 1.5 mL micro test tube at 4 °C, 25,000 × *g*, for 30 min.

Heat the supernatant in thermomixer (Eppendorf thermomixer comfort) at 100 °C for 10 min and centrifuge once again (Eppendorf centrifuge 5417R) in 1.5 mL micro test tube at 4 °C (25,000 × *g*, 30 min).

3.2 Fast Protein Liquid Chromatography

1. Use a solution of 150 mM NaCl in 10 mM Tris–HCl buffer (pH 8.6) as a mobile phase.
2. Adjust the mobile phase flow rate to 4 mL/min.
3. Rinse the column for at least 60 min (240 mL volume) by the mobile phase before separation.
4. During metallothionein separation, use the isocratic elution under ambient temperature of separation column.
5. Inject 2.1 mL of sample (which has to be filtered through 0.45 μm filter before injection) into sample loop (2 mL) using injection syringe (5 mL). Avoid injection of bubbles!
6. Set the UV detector for recording the signal at 254 nm [79]. For typical chromatogram *see* Fig. 3.
7. Fraction collector should be set on collecting of 5 mL fractions between 50 and 60 min of separation (200–240 mL, respectively).
8. For subsequent dialysis choose only these fractions where the peak of MT is notably visible or carry out the SDS-PAGE (see Subheading 3.2) analysis for choosing the specific fractions.

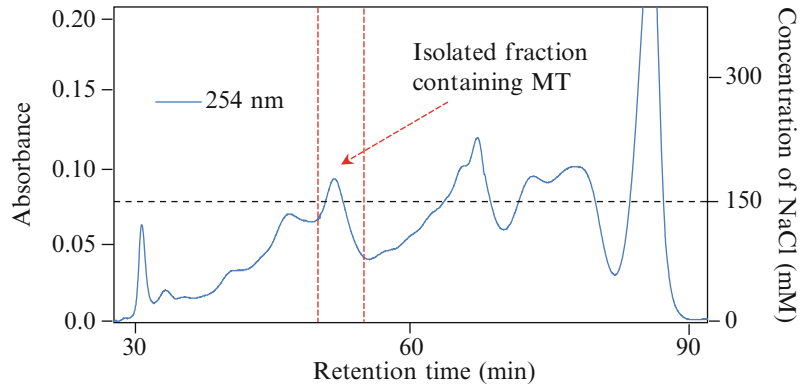

Fig. 3 FPLC chromatogram of real sample of cadmium-treated rabbit liver. Fraction (four fractions of 5 mL) was collected from 50 to 55 min

3.3 Dialysis of FPLC Fraction of MT

1. Dialyze the FPLC fraction by dialysis tubes (Float-A-Lyzer G2, Spectrum labs, USA).
2. Rinse the dialysis tube by distilled water and fill with 5 mL of the fraction.
3. Dialyze the tube in the 2 L beaker filled with distilled water.
4. Replace the water in the beaker four times—every 12 h or until the conductivity of 1.7 μS/cm is reached (this corresponds to the 0.005 mM concentration of ions).

3.4 Lyophilization of Dialyzed MT

1. Lyophilize the dialyzed MT sample (5 mL dialyzed fraction) on glass Petri dish covered by perforated plastic Petri dish using lyophilizer (Christ Alpha 1–2) for 4 h at 1–10 mBar and −50 °C.
2. Transfer the lyophilized MT from the Petri dish by plastic spatula into the storage container.
3. The purity of isolated MT is 5 %. The rest 95 % of the weight is NaCl. Ultrafiltration (Amicon Ultra 3K, Millipore, Merck, Germany) may be used for obtaining more than 99 % purity.
   - Use standard glass and plastic consumables for lyophilization procedure.

3.5 Gel Electrophoresis: Tris–Tricine SDS-PAGE

1. Assemble gel plates into a gel caster. The following volumes are for 1 mm thick gels with dimensions of 8 × 7.3 cm. For other gel thickness or dimensions the total gel volumes should be adjusted.
2. Cast 15 % resolving gel. For one gel mix 5 mL of AA + bis, 3.34 mL of Tris, 0.64 mL of deionized water, 2.12 mL of 50 % glycerol, 100 μL of APS, and 3 μL of TEMED in a beaker. Add the solution in listed order. After addition of APS, mix the

solution by rotation, and after addition of TEMED repeat mixing and immediately transfer the solution between the electrophoretic glasses. Leave approximately 1.5 cm space free. Overlay the gel with 1.5 cm of deionized water and let stand for 45 min.

3. Remove all water from gel surface by pouring off and draining, e.g., with stripe of filter paper, but do not touch the gel.
4. Immediately after removal of water cast 5 % resolving gel. For one gel mix 0.52 mL of acrylamide/bisacrylamide solution, 1 mL of Tris, 2.48 mL water, 40 μL APS, and 2 μL TEMED in a beaker. After addition of APS, mix the solution by rotation, and after addition of TEMED repeat mixing and immediately transfer the solution between the electrophoretic glasses. Insert the comb, and avoid the air bubbles. Let stand for 45 min (*see* **Note 2**).
5. Prepare samples for electrophoresis by mixing of 10 μL of the sample with 5 μL of sample buffer. Heat the samples at 93 °C for 3 min with shaking (300 rpm).
6. Assemble the electrophoresis apparatus. Avoid reusing of running buffer more than twice.
7. Load the samples and standards.
8. Run the electrophoresis at 120 V for 120 min.
9. Stain the gels first with reversible zinc staining, and after destaining stain the gels with silver (*see* **Note 3**).

3.5.1 Zinc Staining

Use 10× gel volume of all solutions, e.g., 50 mL for the abovementioned system.

1. Incubate the gel in diluted solution A (imidazole) for 10 min under mild rotation.
2. Transfer the gel into the diluted solution B (zinc sulfate), and allow the gel to develop for 30–45 s.
3. If you wish, document the gel by scanning or photographing against the dark background or on UV transilluminator.
4. Destain the gel by rinsing three times in destaining solution for 5, 10, and 5 min.

3.5.2 Silver Staining

Use 10× gel volume of all solutions, e.g., 50 mL for the abovementioned system. Use glass trays for all steps, and take new trays for solutions 3, 4, 5, and 6.

1. Incubate the gel in Solution 1 for 45 min.
2. Incubate the gel 3× in Solution 2 for 10 min.
3. Incubate the gel for exactly 1 min in Solution 3.
4. Rinse the gel 2× with 50 mL of deionized water.

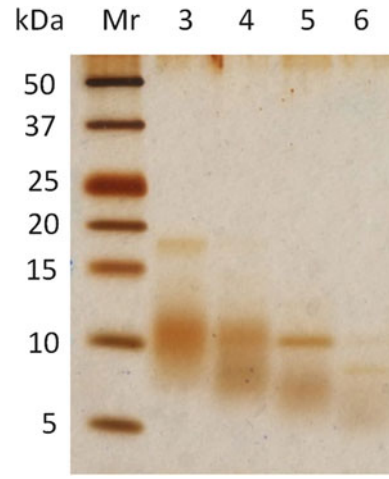

Fig. 4 Typical result of SDS-PAGE analysis of metallothionein-containing fractions from FPLC separation of rabbit liver treated with cadmium

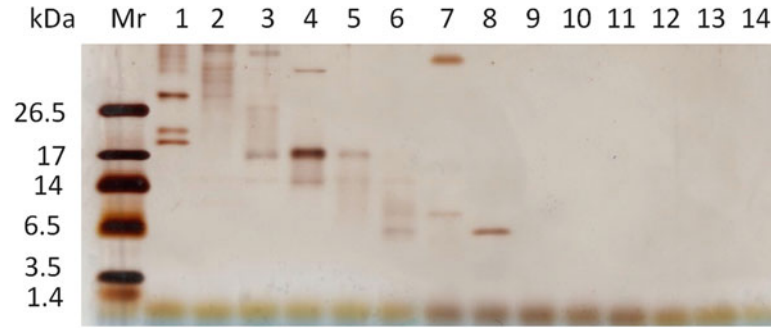

Fig. 5 Typical result of Tris–tricine SDS-PAGE analysis of FPLC fractions of rabbit liver treated with cadmium

5. Incubate the gel for 20 min in Solution 4.
6. Rinse the gel for 20 s in 50 mL of deionized water.
7. Develop the gel in Solution 5 (*see* **Note 4**).
8. Wash the gel 3× for 20 s in 50 mL of deionized water.
9. Stabilize the gel in Solution 6 for at least 10 min. This solution may be used for gel storage.
10. Scan or photograph the gel (Figs. 4 and 5).

3.6 Capillary Electrophoresis of MT Isoforms

1. Prepare MT-1 and MT-2 solutions (0.2 mg/mL) from the stock solution using ultrapure water.
2. Prepare MT-1 and MT-2 solutions (0.1 mg/mL).
3. Prepare the solution of mixture MT-1 and MT-2 by mixing in ratio 1:1 (v/v).

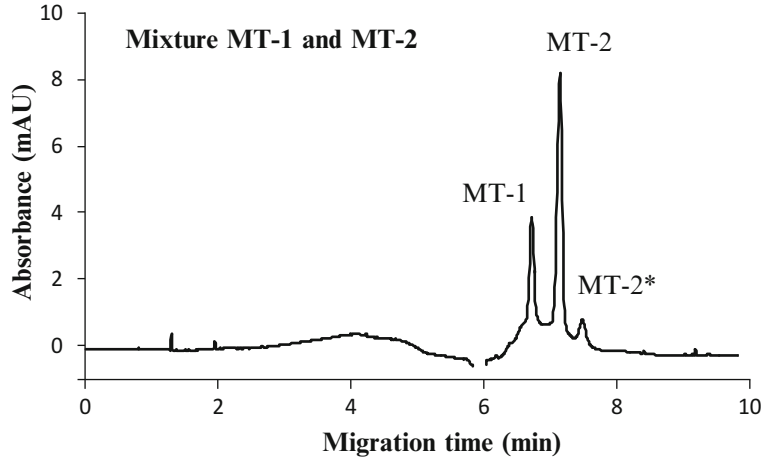

Fig. 6 Electropherogram of the mixture of MT-1 and MT-2 isoforms

4. Analyze by CE under the following conditions: injection—20 s, 3.4 kPa, separation—20 kV for 10 min, capillary—50/57 cm, 75 μm, and UV/VIS detection—254 nm. For typical electropherogram see Fig. 6.

3.7 Heat Treatment of MT

1. Prepare the mixture of MT-1 and MT-2 by mixing 100 μl of MT-1 (0.2 mg/mL) and 100 μL of MT-2 (0.2 mg/mL).
2. Heat the mixture at 99 °C.
3. Take aliquots from the heated sample every 5 min (5, 10, 15, 20 min) and analyze by CE as described in Subheading 3.6 (*see* **Note 5**).

3.8 Chemical Treatment of MT

1. Prepare 100 μl of 0.1 mg/mL MT-1 in 7.5 M urea by mixing the solution of MT-1 (0.2 mg/mL) and 14 M urea solution in volume ratio 1:1.
2. Take aliquots from the heated sample every 15 min (15, 30, 45, 60 min) and analyze by CE as described in Subheading 3.6 (*see* **Note 6**).

3.9 Chip CE

Analyses on an automated microfluidic Experion electrophoresis system (Bio-Rad, USA) are carried out according to the manufacturer's instructions with supplied chemicals (Experion Pro260 analysis kit, Bio-Rad) with the following modifications: the sample buffer is diluted in ratio 1:1 with water, and the concentration of fluorescence dye in gel is lowered to one-half (*see* **Note 7**). Except preparation of calibration curve, 800 ng of MT is used for all analyses.

1. Prepare filtered gel and gel-stain solution.
2. Mix 4 μL of the sample with 2 μL of nonreducing sample buffer, and after 4-min boiling, spin the tubes and add 84 μL of water, vortex, and spin the samples.

3. Prime the chip.
4. Add gel and gel stain into the labelled wells on a chip.
5. Load the sample into the sample wells.
6. Load 6 μL of the Pro260 Ladder included into a ladder well.
7. Immediately put into the electrophoresis system, and start the run (*see* **Note 8**).

4 Notes

1. Zinc staining is compatible with many subsequent procedures like western blotting, amino acid analysis, electroelution, and MALDI-TOF.
2. To obtain best results, cast the gels in advance and store them overnight at 4 °C in dark airtight sealed with plastic wrap. Overnight storage prevents affecting of MT separation by unreacted TEMED and APS.
3. Combination of reversible zinc staining and silver staining enhances MT visualization specifically. The sensitivity is in units of ng per band.
4. Developing time will vary in dependence of protein amount, laboratory temperature, and washing intensity. Excessive rinsing in 6. will result in prolonged development and poor result. Dark or uneven background is caused by contaminations in the trays, insufficient rinsing in 4. or touching the gel with ungloved hands.
5. During 20 min of heat treatment the increase in the peak height of 55 and 65 % should be observed for MT-1 and MT-2, respectively.
6. The increase in the peak height of MT-1 for 32 % and MT-2 for 52 % should be observed.
7. Those modifications dramatically improve capability of the system to detect low amounts of MT.
8. Avoid bubble formation during gel and sample loading. This is best done by reverse pipetting. If bubbles are formed, it is possible to remove them by a small tip or a needle. However, prolonged pipetting or waiting before the analysis causes bad results.

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