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Journal of Chromatography B, 675 (1996) 327–331

JOURNAL OF CHROMATOGRAPHY B: BIOMEDICAL APPLICATIONS

Short communication

Separation of three mouse metallothionein isoforms by free-solution capillary electrophoresis

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Received 15 June 1995; revised 28 August 1995; accepted 5 September 1995

Abstract

We have used free-solution capillary electrophoresis (FSCE) to separate three distinct mouse metallothionein (MT) isoforms, MT-1, MT-2 and MT-3. FSCE was conducted in an uncoated fused-silica capillary (57 cm×50 μm I.D., 50 cm to detector) using 50 m*M* sodium phosphate buffer adjusted to pH 7.0 or 2.0. At neutral pH, each of the three isoform peaks were well resolved from a mixture with the order of migration (MT-1>MT-2>MT-3) related to the net negative charge on the protein. At acidic pH, the migration order was reversed with MT-3 migrating fastest, suggesting MT-3 had a higher net positive charge than MT-2 or MT-1. UV absorbance spectra (190–300 nm) confirmed the presence of Zn in MT-1 and MT-2. MT-3, which was saturated with Cd to stabilize the protein, gave a spectrum characteristic of the Cd–S charge transfer (shoulder at ca. 250 nm). At pH 2.0, the absorbance spectra for all three mouse MTs were characteristic of the metal-free form of the protein (apothionein). Thus, FSCE conducted at neutral pH separates MT isoforms with their metals intact, whereas at pH 2.0, both the Zn and the Cd dissociate from the protein during the run.

Keywords: Metallothioneins; Proteins

1. Introduction

Metallothioneins (MTs) comprise a family of low-molecular-mass, cysteine-rich, heavy metal-binding proteins that are thought to play a fundamental role in cellular metal metabolism [1]. MT isoforms arise from genetic polymorphism which is found in many species. Specific amino acid substitutions among the different MT isoforms can cause changes in their net charge [1]. Since at neutral pH and above MTs exhibit a net negative charge, anion-exchange chromatography is capable of resolving the major charge classes of MTs, designated MT-1 or MT-2 based on their order of elution from the column [2]. Recently, a new MT isoform has been detected and characterized in brain tissue [3,4]. Originally, this protein was called growth inhibitory factor (GIF) because of its ability to inhibit neuronal growth in vitro [3]. However, subsequent analysis of the amino acid [3] and gene [4] sequences revealed distinct MT-like

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0378-4347/96/$15.00
SSDI 0378-4347(95)00379-7

properties, thus giving rise to its new designation as MT-3 [4]. MT-3 elutes from anion-exchange columns after MT-2, indicating that this protein is more negatively charged than MT-2 at alkaline pH [3]. The unusually acidic character of MT-3 compared to other MTs reflects the presence of 3 additional (8 total) glutamic acid residues present in a hexapeptide insert in the COOH-terminal region of the protein [3].

As the number and complexity of identified MT isoforms continues to increase, so too does the need for more rapid and sensitive analytical techniques capable of resolving and quantifying individual isoforms in order to study their specific characteristics and functions. Free-solution capillary electrophoresis (FSCE) conducted in uncoated fused-silica capillaries has been applied to the separation and quantification of MT isoforms from a number of different vertebrate species [5–7]. The objective of this study was to determine if FSCE could successfully separate a mixture of two naturally derived (MT-1 and MT-2) and one recombinant (MT-3) mouse MT isoforms.

2. Experimental[1]

2.1. Instrumentation

FSCE was performed on a P/ACE System 5500 (Beckman, Fullerton, CA, USA). A 50 μm I.D. uncoated fused-silica capillary of 57 cm length (50 cm to the detector) was used. The capillary was housed in a cartridge with liquid cooling to maintain temperature at 25°C. Data were collected and analyzed with System Gold software (Beckman).

2.2. Materials

MT-1 and MT-2 were prepared from zinc-injected mouse liver by sequential gel permeation and anion-exchange column chromatography [2]. Mouse MT-3 cDNA was subcloned into an expression vector (pET3d, Novagen), expressed in *Escherichia coli* and the protein purified by sequential DE-Cellulose, octyl-Sepharose and Sephadex G-50 column chromatography as described previously [8]. Expression was conducted in the presence of 0.4 m*M* $CdSO_4$ because CdMT-3 showed greater stability during purification than did ZnMT-3. Sodium phosphate buffers were prepared from standard reagents or were supplied by Beckman.

2.3. Methods

FSCE was performed under the following conditions: Prior to each run, the capillary was flushed with a wash solution (1 *M* NaOH) followed by water and 50 m*M* sodium phosphate buffer (pH 2.0 or 7.0) for 2.0 min each. The MT isoforms were dissolved in deionized water at an estimated final concentration of 1.0 mg/ml. Mixed samples were loaded into the capillary by pressure injection at 3.45 kPa. An injection time of 2.0 s was used for separations conducted at pH 7.0 which constituted an injection volume of 2.4 nl containing an estimated 2.4 ng of MT. The injection time was increased to 5.0 s for separations conducted at pH 2.0 since under these conditions a decrease in absorbance at 200 nm was anticipated [1]. The run was initiated by applying 30 kV across the capillary. MT isoforms were identified by monitoring their absorbance at 200 nm using photodiode array detection. Absorbance spectra (190–300 nm) were obtained at the apex of each MT isoform peak.

3. Results and discussion

Three major charge classes of mouse MT (MT-1, MT-2 and MT-3) were well resolved from a mixture at neutral pH in under 7 min (Fig. 1). A comparison of the migration times for the three mouse MT isoforms clearly shows that at neutral pH, MT-3 carries the highest net negative charge followed by MT-2 and MT-1. The presence of 7 divalent metal ions bound to the protein via thiolate complexes imparts 6 negative charges which together with the differing charges from the polypeptide chain itself determines the net charge of the MT isoform [1]. Since it has been reported that the MT-1 protein

[1]Mention of a trade name, proprietary product or specific equipment does not constitute a guarantee or warranty by the US Department of Agriculture and does not imply its approval to the exclusion of other suitable products.

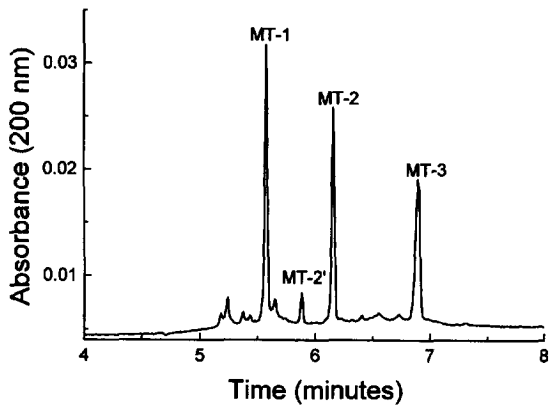

Fig. 1. Typical electropherogram of a mixture of mouse MTs which were each dissolved at a concentration of 1.0 mg/ml in deionized water and loaded into the capillary by pressure injection for 2.0 s. A 57 cm×50 μm I.D. (50 cm to detector) fused-silica capillary was used. The running buffer was 50 m*M* sodium phosphate, pH 7.0 and the running voltage was 30 kV. MT isoform peaks were detected by their absorbance at 200 nm. The MT-1, MT-2 and MT-3 peaks are indicated.

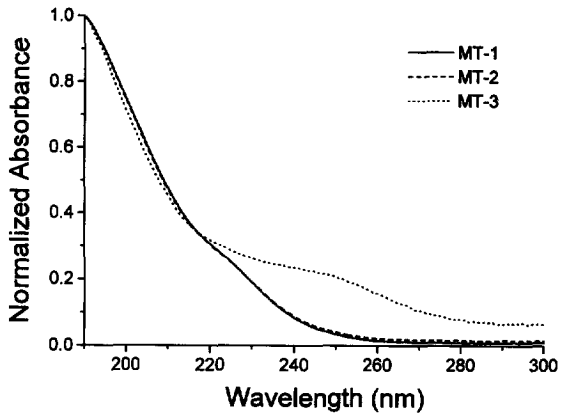

Fig. 2. Normalized UV absorbance spectra obtained using photodiode array detection for MT isoform peaks separated by FSCE. Each line represents a scan between 190 and 300 nm at 1 nm intervals with the data collected at 4 points per second. The scans depicted represent those obtained at the apex of each mouse MT isoform peak detected in Fig. 1. Those scans corresponding to the MT-1, MT-2 and MT-3 peaks are so designated.

carries a net −2 charge and MT-2, a net −3 charge at pH 8.6 [1], the migration behavior of the mouse MT-3 isoform under the conditions used in this separation is consistent with it having a net negative charge below −3. Although a net −4 charge has been reported for human metal-free (apo-) MT-3 [3], the actual net charge for a particular MT-3 protein is likely to reflect species-specific differences in its amino acid sequence and metal composition [9]. In fact, FSCE may be a useful technique to accurately determine net charge of MT isoforms at a given pH since the separation is based on differences in charge/mass ratio. A minor peak migrating between MT-1 and MT-2 (see MT-2′ in Fig. 1) originated from MT-2 (data not shown) and may represent additional heterogeneity in this isoform charge class. There have been several reports of microheterogeneity in both the rat and mouse zinc-induced MT-2 isoform [10–12]. Whether or not this peak represents a distinct isoform or is the product of post-translational modification of MT-2 remains to be established.

The utility of photodiode array detection of MT isoforms is depicted in Fig. 2 which shows absorbance spectra (190–300 nm) obtained at the apex of each MT isoform peak separated by FSCE (Fig. 1). The MT-2′ peak contained an insufficient amount of material to obtain an adequate absorbance spectrum. Useful information concerning the metal composition of each isoform can be obtained because of the unique charge-transfer transitions characteristic of the metal-thiolate bond [1]. The shoulder at 230 nm seen in the spectra for MT-1 and MT-2 is indicative of ZnMT, whereas the shoulder at 250 nm observed in the MT-3 spectrum is indicative of CdMT (Fig. 2). Although the spectrum obtained for MT-3 is indicative of a CdMT, the existence in the MT-3 sample analyzed of some ZnMT-3 or the mixed metal form (viz., Cd,ZnMT-3) cannot be excluded.

At pH 2.0, the order of migration for the three mouse MT isoforms reverses from that observed at neutral pH (Fig. 3). At low pH (below the p*I*), MTs exhibit a net positive charge, the magnitude of which depends on the number of basic amino acid residues contained in the protein sequence. The migration order reversal indicates that at pH 2.0, MT-3 is more positively charged than MT-2 or MT-1. A peak corresponding to MT-2′ was also seen. Complete reversal in migration order observed for mouse MT isoforms run at pH 2.0 vs. 7.0 may be somewhat species-specific since this behavior was not observed when rabbit liver and horse kidney MTs were

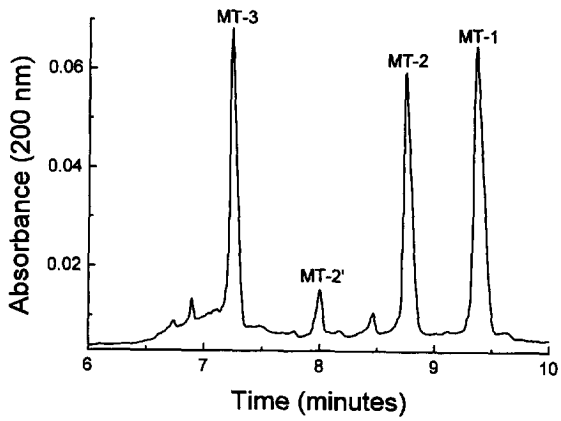

Fig. 3. Typical electropherogram of a mixture of mouse MTs which were separated as described in the legend to Fig. 1 except that the running buffer was 50 m*M* sodium phosphate pH 2.0 and a 5.0 s sample injection time were used. The MT-1, MT-2 and MT-3 peaks are indicated.

subjected to the identical separation conditions (data not shown). The absorbance spectra (190–300 nm) for all three mouse MT isoforms obtained at pH 2.0 were nearly identical (Fig. 4). The spectra depicted in Fig. 4 are very similar to those obtained previously for apoMTs (apothioneins) by direct spectroscopic measurement [13,14] and thus indicate that at pH 2.0, the metals bound to the MTs were removed such that the isoform peaks separated and detected by FSCE represent apothioneins.

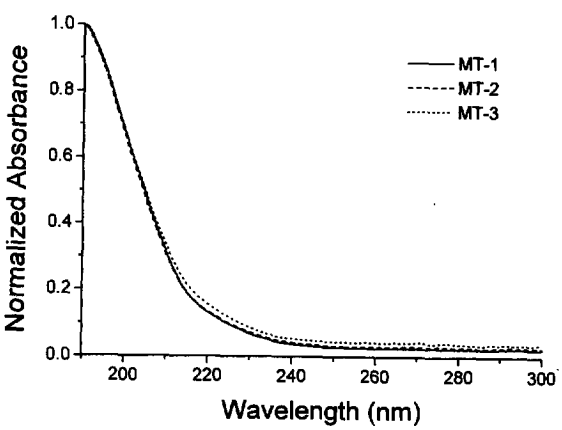

Fig. 4. Normalized UV absorbance spectra obtained using photodiode array detection as described in the legend to Fig. 2 for MT isoform peaks separated by FSCE. The scans depicted represent those obtained at the apex of each mouse MT isoform peak detected in Fig. 3. Those scans corresponding to the MT-1, MT-2 and MT-3 peaks are so designated.

4. Conclusions

This study demonstrates the rapid and complete resolution of three mouse MT isoforms (MT-1, MT-2 and MT-3) from a mixture using FSCE conducted at neutral or acidic pH. At pH 7.0, MT-3 was found to have the highest net negative charge, followed by MT-2 and MT-1. At pH 2.0, MT-3 exhibited a higher net positive charge than MT-2 or MT-1. Absorbance spectra, obtained using photodiode array detection, indicate that the MT isoforms separated at neutral pH retain their bound metals, whereas at low pH cadmium and zinc dissociate from the proteins such that the isoforms separated are actually apothioneins. FSCE with photodiode array detection represents a useful analytical technique for the rapid separation and characterization of MT isoforms. Application of this technique to the analysis of MT isoforms in more complex matrices such as tissue extracts is currently being explored.

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