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Short communication

Rapid identification of metallothionein isoforms in liver cytosol fraction by capillary zone electrophoresis using EDTA

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Abstract

Identification of metallothionein (MT) isoforms on capillary zone electrophoresis (CZE) analysis was studied using a linear polyacrylamide-coated capillary at pH 7.4 and EDTA. The CZE system was able to separate standard (purified and commercially available) MT specimens into their isoforms within 10 min. The peaks of MT-1 and MT-2 isoforms disappeared on addition of EDTA to the specimen, and the disappearance was shown to be time-dependent and dose-dependent, although the reason why the peaks decreased is still unclear. A heat-treated cytosol fraction prepared from Zn-injected mouse liver showed many major and minor peaks on CZE analysis. Two major peaks were identified to be MT-1 and MT-2, respectively, by co-injection with the purified MT isoforms. When EDTA was added to the cytosol fraction, the two major peaks, MT-1 and MT-2, and three other minor peaks disappeared time-dependently. Therefore, each MT isoform in the cytosol fraction can be identified by the addition of EDTA, also the peaks are identified by the corresponding migration times of purified MTs. Unknown substances like MT sub-isoforms may also be detected, although this question warrants clarification. From these results, it was concluded that the addition of EDTA is useful for identification of MT isoforms in cytosol fractions on CZE analysis.

Keywords: Metallothionein; EDTA

1. Introduction

Metallothionein (MT) is a protein of about 7000 Da that contains a high amount of cysteine and bonds with several heavy metal ions such as Zn, Cu and Cd. All cysteine residues occur in the reduced form and are coordinated to the metal ions through mercaptide bounds, giving metal–thiolate complexes and metal–thiolate clusters [1]. Mammalian MT has been mainly subdivided into two classes, MT-1 and MT-2, although MT has four isoforms. They can be resolved by size-exclusion chromatography using a column with cation-exchange properties [2] and by anion-exchange chromatography [3]. Recently developed techniques in analytical chemistry, however, have revealed that this classification is insufficient. Both capillary zone electrophoresis (CZE) using an

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uncoated capillary and reversed-phase high-performance liquid chromatography (RT-HPLC) [4–6] have separated the isoforms into several sub-isoforms. Chassaigne and Lobinski [7] suggested that MT isoforms were identified by using HPLC–ion spray MS, and Knudsen et al. [8] also identified MT isoforms using CE-electrospray MS. However, the methods need a specific instrument. In the CZE system using an uncoated capillary, it was shown that the electrophoresis conditions, such as buffer composition including additives, buffer concentration and buffer pH, are important for obtaining high-separation efficiency [8–11]. These conditions probably result in a lowering of the interaction between proteins and the inner wall of capillary. In contrast to the current use of an uncoated capillary for CZE separation of MT isoforms, a capillary with a neutral polymer coating has been solely used until recently. The reports of Minami and co-workers [12–16] were the first to successfully separate the isoforms using a polyacrylamide-coated capillary, although there is one paper in which the assignment of peaks was not performed [5]. A neutral polymer coating of the inner walls of a capillary, as first described by Hjertén [17], often yields significant improvements in resolution of proteins, and makes it possible to separate proteins even at physiological pH [18–20] where protein adsorption is often a problem. As UV absorption is generally used for detection of peaks in CZE analysis, and as the specimens needed for CZE analysis have very little volume, it is difficult to identify peaks on CZE analysis. We previously identified the MT isoforms using CZE analysis by comparing their migration times with those of purified MT isoforms [12]. However, the matrix components in the specimen, the viscosity and the life span of the capillary affect the migration time. By resolving these problems, the aim of present study was to develop a rapid and simple identification method with high-resolution efficiency for detecting isoforms of MT in mouse liver cytosol fractions by CZE analysis. For this purpose the effect of EDTA on the electropherogram of MT isoforms is evaluated by CZE using a polyacrylamide-coated capillary, as there are several reports presenting the relationship between metal contents in MT and UV absorption [21,22].

2. Materials and methods

2.1. Materials

N-2-Hydroxyethylpiperazin-*N′*-2-ethanesulfonic acid (Hepes) and EDTA-Na_2 were purchased from Dojindo Laboratories (Kumamoto, Japan). Acrylamide, sucrose, *N,N,N′,N′*-tetramethylethylenediamine (TEMED), ammonium persulfate and tris (hydroxymethyl) aminomethane (Tris) were from Wako Pure Chemicals (Osaka, Japan). 3-Methacryloxypropyltrimethoxysilane was from Shin-Etsu Silicon Chemicals (Tokyo, Japan). Rabbit liver MT-1 was purchased from Sigma (St. Louis, MO, USA). All other chemicals were of analytical grade and obtained from Wako Pure Chemicals. The fused-silica capillary tube (I.D. 75 μm; O.D. 375 μm) was obtained from Otsuka Electronics (Osaka, Japan).

2.2. CZE analysis

CZE with a polyacrylamide-coated capillary was performed on a Quanta 4000 capillary electrophoresis system (Nihon Waters, Tokyo). The capillary was coated according to the previous report [19]. The electrophoresis was carried out with a running buffer of 50 m*M* Hepes–Tris, pH 7.4, at 26°C at a constant voltage of −20 kV on a capillary with a total length of 33 cm (effective length of 25 cm). The samples were introduced from the cathode end of the capillary by gravity. Separations were monitored at 214 nm.

2.3. Preparation of purified MT isoforms

Purified mouse MT-1 and MT-2 isoforms were prepared from the livers of mice injected with cadmium chloride according to the method of Kimura et al. [23].

2.4. Preparation of mouse liver cytosol fraction

Mice were subcutaneously injected with 50 mg/kg of zinc in the form of zinc sulfate. Twenty-four hours after injection, the mice were decapitated and the livers were removed. The liver samples were frozen until required. The liver was homogenized to

make a 10% homogenate with a 0.25 *M* aqueous sucrose solution. The homogenate was immediately centrifuged at 700 *g* for 10 min, and the supernatant was centrifuged at 100 000 *g* for 30 min. Part of the supernatant was frozen and the other part was incubated in a boiling water bath for 1 min, and again centrifuged at 8000 *g* for 10 min. Aliquots of the heat-treated preparation were placed in microtubes for incubation with EDTA.

2.5. Incubation with EDTA and specimens

For observation of peaks of purified MT-1 and MT-2 isoforms, 1 μl of 20 m*M* EDTA was incubated with 9 μl of each MT isoform (0.05 mg/ml) at room temperature under an air aerobic condition. When the effect of EDTA on commercial standard MT-1 isoform was observed, 2 μl of 2, 10, and 20 m*M* EDTA were added to 10 μl of the MT-1 (0.5 mg/ml). For observation of the effects of EDTA on MT isoforms in cytosol fraction, 2 μl of EDTA (20 m*M*) were added to 10 μl of each heat-treated cytosol specimen obtained from Zn-injected mouse liver.

3. Results

3.1. Effect of EDTA on electropherogram of purified MT-1 and MT-2 isoforms

The effects of EDTA on purified MT-1 and MT-2 isoforms are shown in Fig. 1. Purified MT-1 migrated at 7.3 min (Fig. 1a) and MT-2 at 5.2 min (Fig. 1e) on CZE analysis. When EDTA was added to the purified MT-1, the height of peak of the MT-1 decreased after 2 min (Fig. 1b), and after12 min the peak disappeared (Fig. 1c). In contrast, the peak of MT-2 decreased slightly 2 min after the addition of EDTA (Fig. 1f). A time-dependent decrease was observed (Fig. 1g), and the peak disappeared at 35 min (Fig. 1h).

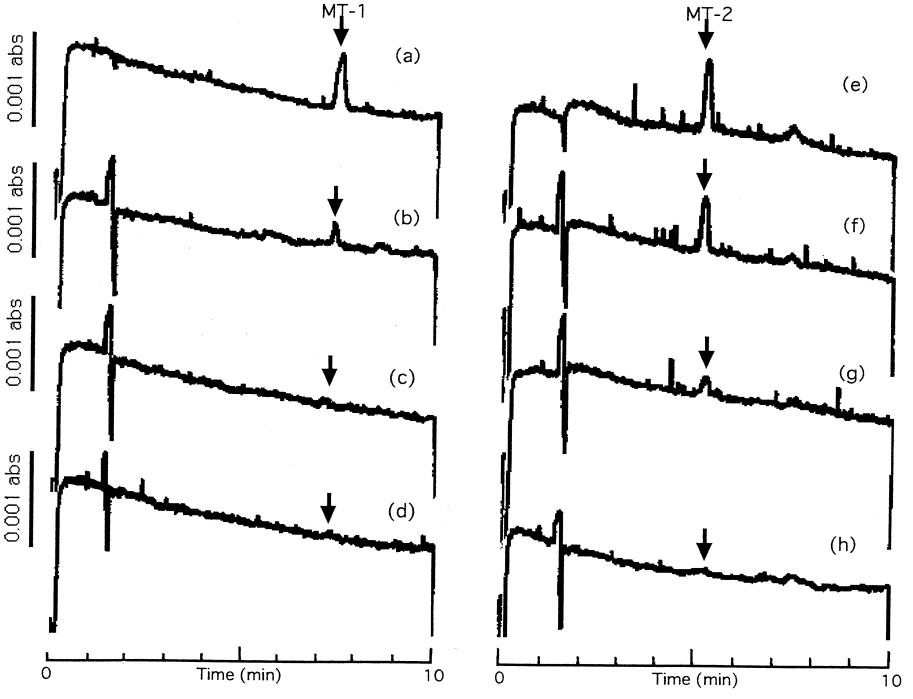

Fig. 1. Effect of EDTA on electropherograms of purified MT isoforms. (a) Purified MT-1. (e) Purified MT-2. Each isoform was incubated with 20 m*M* EDTA for 2 min (b,f), 12 min (c,g), and 35 min (d,h), and the specimen was then loaded onto the column for 10 s by gravity.

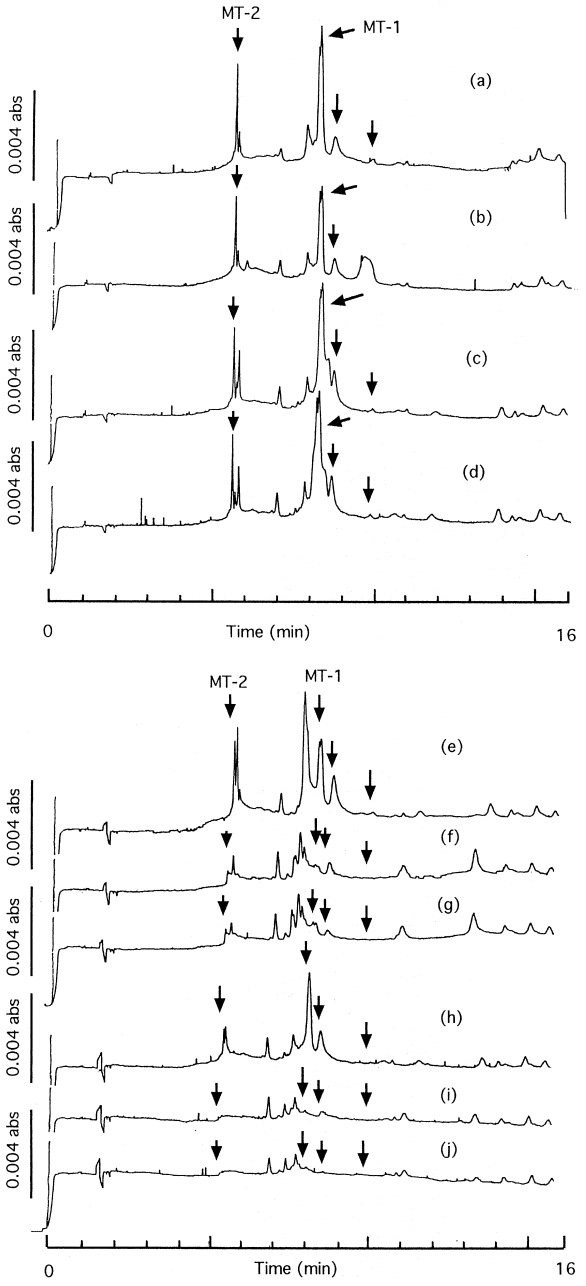

Fig. 2. Effect of EDTA concentrations on commercial standard MT-1. (a) Standard MT-1. MT-1 was incubated with 2 m*M* EDTA for 2 (b), 20 (c) and 40 min (d). MT-1 was incubated with 10 m*M* EDTA for 2 (e), 20 (f) and 40 min (g). MT-1 was incubated with 20 m*M* EDTA for 2 (h), 20 (i) and 40 min (j).

3.2. Effect of dosage of EDTA on electropherogram of commercial standard MT-1

Dose-dependent decreases of MT peaks by EDTA using commercial standard MT-1 are shown in Fig. 2. In commercial standard MT-1, there were several peaks, including MT-2 peak, separated on CZE analysis (Fig. 2a). When 2 m*M* EDTA was added to the commercial standard MT-1, no peaks decreased until 40 min (Fig. 2b–d). However, both MT-1 and MT-2 peaks decreased slightly 2 min after the addition of 10 m*M* EDTA (Fig. 2f). After 20 min, the peak of MT-1 disappeared, but a small peak of MT-2 still remained until 40 min (Fig. 2g). The other two minor peaks shown in standard MT-1 on CZE analysis also decreased time-dependently after the addition of EDTA (Fig. 2e–g). Furthermore, both peaks of MT-1 and MT-2 decreased 2 min after the addition of 20 m*M* EDTA (Fig. 2h) and disappeared at 20 min (Fig. 2i). The other two minor peaks also decreased in a time-dependent manner (Fig. 2h–j).

3.3. Effect of EDTA on peaks of MT isoforms in cytosol fraction

There were many major and minor peaks in the heat-treated cytosol fraction of Zn-injected mouse liver on CZE analysis (Fig. 3a). From the migration times of purified MT-1 and MT-2 isoforms, the peaks migrating at 8.2 and 5.9 min were identified as MT-1 and MT-2, respectively. The peak migrating at 8.2 min decreased 2 min after the addition of EDTA (Fig. 3b), and disappeared after 15 min (Fig. 3c–e). In contrast, although the peak migrating at 5.9 min decreased after 2 min, the peak height did not change until 90 min (Fig. 3a–e). There were three other peaks, which disappeared after the addition of EDTA in a time-dependent manner (Fig. 3a–e).

4. Discussion

CZE is an effective method for separation of substances because of its associated rapid analysis, microsampling, and high resolution. It is difficult, however, to identify the peaks after separation on CZE analysis. Migration times corresponding with those of the standard are generally used for identifi-

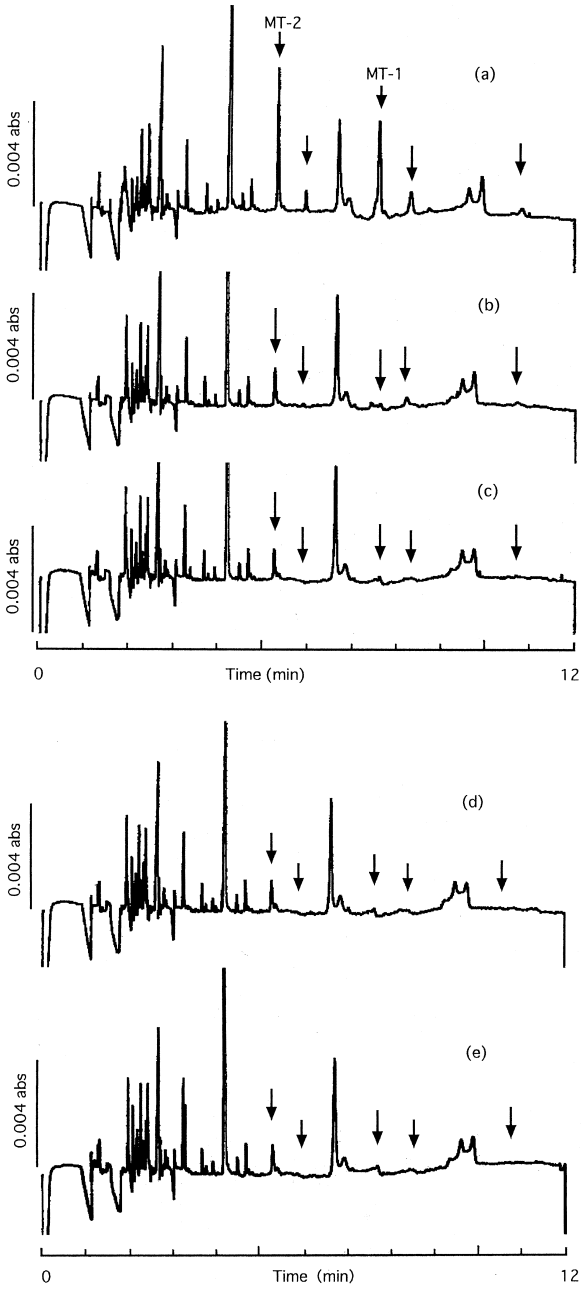

Fig. 3. Effect of EDTA on heat-treated cytosol fraction of Zn-injected mouse liver. (a) Heat-treated cytosol fraction of Zn-injected mouse liver. The specimen was incubated with EDTA for 2 (b), 15 (c), 30 (d) and 90 min (e).

cation, but the migration times are not reliable because they depend on the components of the matrix in solution, the viscosity of solution and the life span of the capillary. Lu et al. [24] developed the method of CZE-ICP-MS for detection of MT isoforms, and Wittrisch et al. [25] used a proton-induced X-ray emission detector for the identification of MT isoforms after separation by CZE analysis. Recently, Knudsen and Beattie [26] developed a method for measurement of the concentration of MT isoforms by using an on-line solid-phase extraction-CZE system. However, these methods are not available generally, because they need a specific instrument and high volumes for sampling. In addition, it is difficult to identify sub-isoforms of MT after separation by CZE analysis. Virtanen et al. [11] observed sub-isoforms of MT on CZE analysis, however, we could not identify MT sub-isoforms after separation on CZE analysis, as we did not have the purified MT sub-isoforms corresponding with the migration times of peaks of cytosol fraction after separation by CZE analysis.

We previously [13] observed that the peaks of MT isoforms decreased on CZE analysis after the addition of excess Cd to the MT specimen, although the mechanism was not clear. In the present study, we added EDTA to MT specimens, expecting the peaks of MT isoforms to change with Cd addition for identification of the MT isoforms. As shown in the present results, the peaks confirmed as MT-1 and MT-2, from the migration times corresponding with those of the purified MT isoforms, decreased in a dose-dependent and time-dependent manner after the addition of EDTA. Therefore, the MT-1 and MT-2 isoforms in mouse liver cytosol fraction can be identified on CZE analysis with or without EDTA. The reason why the peaks decrease after addition of EDTA is still unclear, although UV absorption of MT depends on the contents of metals [21,22]. The loss of metal–mercaptide transitions and/or polymerised adducts may decrease the absorption of UV after the addition of EDTA. The peak of MT-2 in the heat-treated cytosol fraction of Zn-injected mouse liver did not disappear completely until 90 min after the addition of EDTA (Fig. 3). An unknown substance that is not a MT isoform may have migrated at the same time, corresponding with the peak of MT-2 isoform. Furthermore, several minor peaks of

heat-treated specimens of Zn-injected mouse liver on CZE analysis decreased with MT isoforms after the addition of EDTA. Beattie et al. [27] observed that MT-2 isoform had both *N*-acetylate and non-acetylate forms after separation by CZE. Unknown heat-stable metal-bound substances like MT sub-isoforms may be detected in the present study, but this question warrants clarification.

In conclusion, the addition of EDTA is useful for the identification of MT isoforms in cytosol fraction on a CZE analysis.

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