ELSEVIER

Journal of Chromatography B, 736 (1999) 185–190

JOURNAL OF CHROMATOGRAPHY B

www.elsevier.com/locate/chromb

Identification of metallothionein isoforms on capillary zone electrophoresis by adding anti-metallothionein antibody

Kanenobu Kubo[a], Yuko Sakita[a], Yuko Okazaki[a], Noriko Otaki[b], Masami Kimura[c], Takeshi Minami[d,*]

[a]*Faculty of Pharmaceutical Sciences, Kinki University, 3-4-1 Kowakae, Higashi-Osaka, Osaka 577, Japan*
[b]*Department of Occupational Disease, National Institute of Industrial Health, Kawasaki, Kanagawa 214, Japan*
[c]*Department of Molecular Biology, School of Medicine, Keio University, Tokyo 160, Japan*
[d]*Department of Life Sciences and Program Processing, Toyo-Oka Junior College, Kinki University, 160 Tobera, Toyo-Oka, Hyogo 668-8580, Japan*

Received 2 June 1999; received in revised form 9 September 1999; accepted 6 October 1999

Abstract

The aim of this study was to identify metallothionein (MT) isoforms in mouse liver by using capillary zone electrophoresis (CZE). Purified MT-1 and MT-2 isoforms were completely separated by CZE using a polyacrylamide-coated tube at physiologic pH. There were two peaks in the cytosol fraction prepared from zinc-injected mouse liver, in which the migration times corresponded with those of purified MT-1 and MT-2 isoforms. When anti-MT monoclonal antibody was added with the purified MT-1 or MT-2 solution, the peaks decreased. Furthermore, the two peaks in the cytosol prepared from Zn-injected mouse liver decreased in a time-dependent manner from the electropherogram after the addition of the antibody. Therefore, those peaks were identified as MT-1 and MT-2 isoforms, respectively. In conclusion, the addition of anti-MT monoclonal antibody to the cytosol fraction of tissues is an effective method for identification of MT isoforms after separation using CZE.

Keywords: Metallothionein isoforms

1. Introduction

Capillary zone electrophoresis (CZE) is useful for the separation of substances in specimens. CZE has several advantages, such as rapid analysis, high resolution, and microsampling, in comparison with the other methods [1,2]. However, CZE also has several drawbacks. One major problem is how to identify the constituents. Since a micro volume of specimen is used for CZE analysis, methods for detection are limited. UV absorption is generally used for detection, and the method for identification used is the migration time corresponding with that of the standard specimen. The metallothionein (MT) protein is inducible by various stimuli like metals and stress [3], and it has four major isoforms and several sub-isoforms [4–6]. However, it is difficult to identify the isoforms in tissue samples on CZE analysis. Although, we previously identified the MT isoforms using CZE analysis by comparing their

*Corresponding author. Tel.: +81-796-22-6361; fax: +81-796-22-6366.
E-mail address: minamita@hk.sun-ip.or.jp (T. Minami)

0378-4347/99/$ – see front matter
PII: S0378-4347(99)00461-2

migration times with those of purified MT isoforms [7], the matrix components in the specimen, the viscosity, and the life span of the tube affect the migration time. In addition, it is very difficult to identify sub-isoforms [8] from their migration times without purified sub-isoforms. Therefore, we attempted at first to identify the MT isoforms by adding anti-MT monoclonal antibody to the CZE column.

2. Experimental

2.1. Materials

The purified MT-1 and MT-2 isoforms were prepared from the liver of Cd-injected mice. The details of purification and separation were described in our previous report [7]. The anti-MT monoclonal antibody (Dako-MT, E9) solution was purchased from Dako (CA, USA). The concentration of immunogloblin G in the solution was 122 μg/ml and a large amount of albumin was added in the solution for stabilization. Tris(hydroxymethyl)aminomethane (Tris) was purchased from Wako Pure Chemicals (Osaka, Japan), and *N*-2-hydroxyethylpiperazin-*N′*-2-ethanesulfonic acid (HEPES) from Dojindo Laboratories (Kumamoto, Japan). A fused-silica capillary tube (75 μm I.D.) was prepared by Otsuka Electronics (Osaka, Japan). The other reagents were purchased from Wako Pure Chemicals.

2.2. Animals

Male ddY mice, 6-weeks-old, were obtained from Japan SLC (Shizuoka, Japan) and housed for 1 week before the experiment. Animals were fed a standard diet (MF, Oriental Yeast, Tokyo, Japan) with free access to tap water.

2.3. Zone electrophoresis on a polyacrylamide-coated tube

We used a 33-cm polyacrylamide-coated capillary tube. The coating method was described in our previous report [9]. The tube was installed in a capillary electrophoresis system (Waters Quanta 4000, Japan Millipore, Tokyo). HEPES–Tris buffer (50 m*M*, pH 7.4) served as the running buffer, and the samples were loaded onto the column by gravity. The inlet polarity was that of cathode. The analysis was performed at 20 kV and 26±1°C. The MT isoforms were monitored at 214 nm.

2.4. Animal treatment

The mice were given subcutaneous injections of 50 mg/kg of zinc in the form of zinc sulfate. Twenty-four hours later, the mice were decapitated and their livers were removed. The livers were homogenized to make a 10% homogenate with 100 m*M* Tris–HCl buffer at pH 7.4. After centrifugation at 100 000 *g* for 60 min, the cytosol fraction was obtained.

2.5. Addition of purified MT isoforms into liver cytosol fraction

The cytosol fraction was prepared from Zn-injected mouse liver and boiled at 100°C for 1 min. The supernatant was obtained after a centrifugation at 10 000 *g* for 5 min. The supernatant was passed through a 0.45-μm filter and added to the same volume of purified isoform (0.1 mg/ml) before CZE analysis.

2.6. Reaction of purified MT isoforms with antibody

The solution of anti-MT monoclonal antibody was passed through the column of an affinity chromatography kit for isolation of IgG (MabTrap GII, Pharmacia Biotech, Sweden). The IgG fraction was centrifuged by using a molecular cut filter (Ultrafree-MC Centrifugal Filter Units, 10 000 MWL, Japan Millipore, Tokyo) and high molecular weight fraction was collected. After desalting, lyophilized powder was dissolved with 2.5 μl of each purified MT isoform (0.1 mg/ml) and 2.5 μl of distilled water, and reacted for 30 min at room temperature. Finally, 20.7 μg of IgG was included in the reacting solution.

2.7. Reaction of cytosol fraction with antibody

Liver cytosol fraction obtained from Zn-injected mouse was passed through a 0.45-μm filter. A 2 μl

aliquot of filtrated solution was added to 18 μl of antibody solution (122 μg IgG/ml) without heat treatment and applied to CZE analysis time-dependently.

3. Results

Fig. 1 shows an electropherogram of a cytosol fraction of Zn-injected mouse liver after heat treatment. There were many peaks detected during 10 min after starting. Peaks were well separated each other at a neutral pH condition and the baseline was relatively stable (Fig. 1a). When purified MT-1 was added to the boiled cytosol solution, one peak migrated at 7.2 min increased (Fig. 1b). The other peak migrated at 5.0 min increased after the addition of purified MT-2 to the cytosol solution (Fig. 1c).

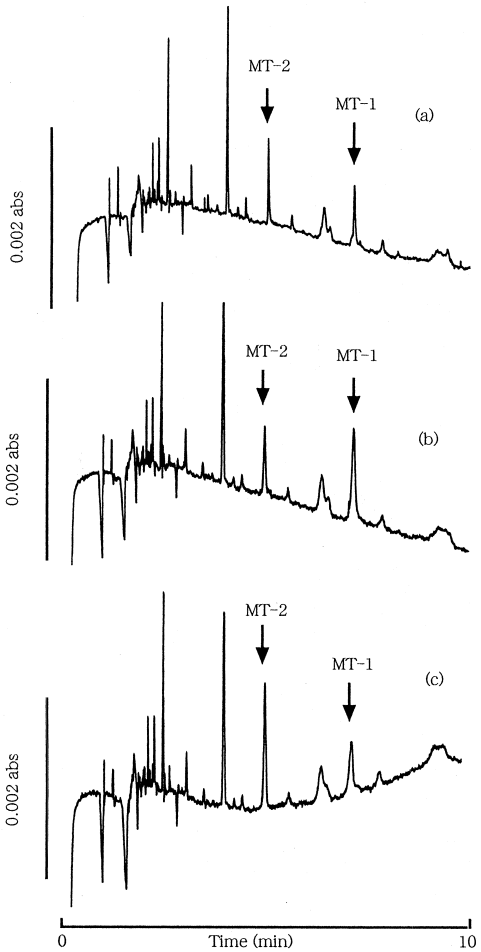

Fig. 1. Electropherogram of the cytosol fraction of Zn-injected mouse liver after boil treatment. (a) Boiled cytosol with distilled water, (b) boiled cytosol with purified MT-1 isoform (0.1 mg/ml) and (c) boiled cytosol with purified MT-2 isoform (0.1 mg/ml). The specimen was loaded onto the column for 5 s by gravity and monitored at 214 nm.

Purified MT-1 showed a single peak at 7.1 min (Fig. 2a). The peak disappeared 30 min after the addition of the IgG fraction prepared from the anti-MT monoclonal antibody (Fig. 2b). Purified MT-2 as well as MT-1 was detected as a peak at 5.0 min and very small peak was also detected at 7.1 min (Fig. 2c). Thirty minutes after the addition of the IgG fraction to the purified MT-2 solution, the peak migrated at 5.0 min decreased and the peak migrated at 7.1 min disappeared (Fig. 2d).

When anti-MT monoclonal antibody solution was directly applied to CZE analysis, one large and two small peaks were shown during 13 min after starting (Fig. 3a). On the contrary, non-boiled cytosol solution prepared from Zn-injected mouse liver showed one large and several small peaks including two peaks migrated at 5.0 and 7.1 min (Fig. 3b). Two minutes after the addition of antibody to the cytosol solution, the peak shown at 5.0 min migrated a little faster than the original peak. On the contrary, the peak shown at 7.1 min was overlapped with the former small peak of the antibody, and the peak area increased (Fig. 3c). The peak of cytosol solution that had migrated at 5.0 min decreased gradually in the time-dependent manner after the addition of antibody, but had not disappeared until 60 min. After 15 min, the peak of cytosol that had migrated at 7.1 min also decreased in comparison with the peak shown 2 min after the addition of antibody. The peak area of cytosol 30 min after the addition of the antibody was not different from the area shown 60 min after (Fig. 3d and e).

4. Discussion

MT has several functions including the storage of essential metals, detoxification of heavy metals, and the scavenging of free radicals [3]. MT has four major isoforms and several sub-isoforms. The structure of MT-1 is very similar to MT-2, and these

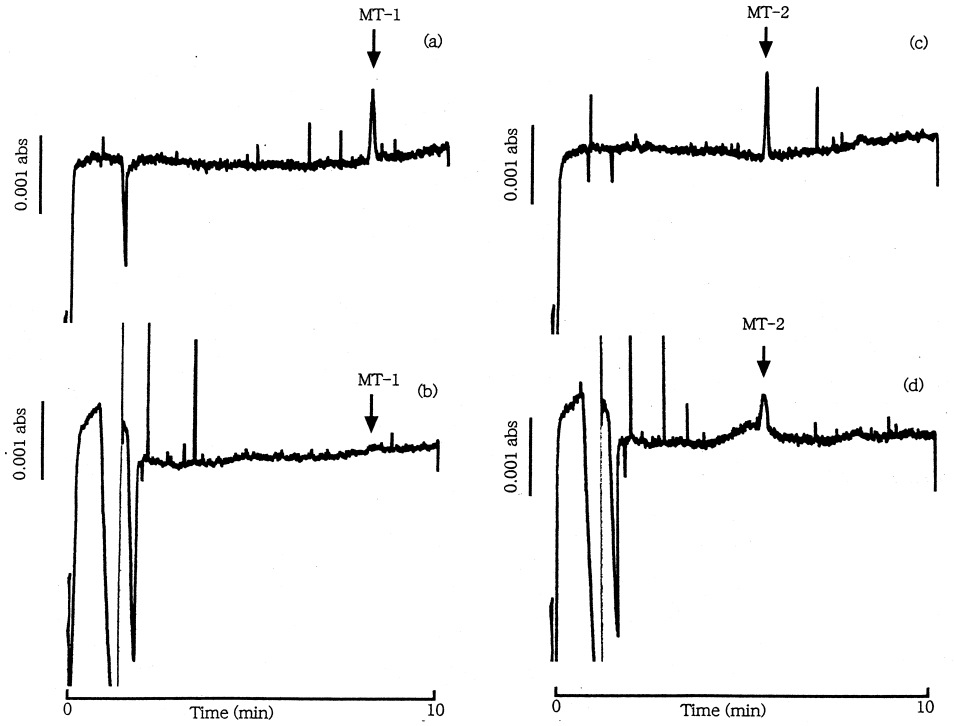

Fig. 2. Effect of the addition of the IgG fraction prepared from the antibody on the CZE chromatogram of purified MT isoforms. (a) Purified MT-1 isoform, (b) purified MT-1 isoform with IgG, (c) purified MT-2 isoform and (d) purified MT-2 isoform with IgG. The specimen was loaded onto the column for 10 s by gravity and monitored at 214 nm.

isoforms are induced coordinately in most organs [5,10]. Nevertheless, Kobayashi and Sayato-Suzuki [11] reported that MT-1 functioned mainly in metal metabolism and MT-2 in cell growth. Kumari et al. [12] also reported that MT-1 had a higher affinity for free radicals than MT-2. Therefore, it is of interest to distinguish between the actions of MT-1 and MT-2 isoforms in the organs. Although it is known that MT has sub-isoforms [6], the functions of its sub-isoforms are still not clear because it is very difficult to identify them and to measure their concentrations in organs. MT-1 and MT-2 isoforms are generally measured using the ELISA method [13] or atomic absorption spectrometry, fluorometry, and ICP–MS methods after separation by HPLC [14–17]. CZE analysis is also used for the separation of MT isoforms [18], and Richards and Beattie [19] developed the method of using HPLC in combination with CZE. Recently, Lu et al. [20] developed the method of CZE–ICP–MS for the detection of each isoform, while Wittrisch et al. [21] used a proton-induced X-ray emission detector for the identification of MT isoforms separated by CZE analysis. However, we have only an UV detector for the identification of each isoform. It is necessary to use the previous methods for the identification of high concentrations of MT isoforms using CZE analysis. Interestingly, Knudsen and Beattie [22] detected MT isoforms by using an on-line solid-phase extraction–CZE system, and measured the concentrations of MT isoforms in the sheep fetal liver from their UV absorption. We separated MT isoforms, extracted from mouse liver, at a neutral pH by CZE using a polyacrylamide-coated tube. We previously identified the isoforms from their migration times corresponding with those of the purified isoforms. [7]. As shown in Fig. 1, the peaks of the MT isoforms in the cytosol fraction were confirmed from the migration times corresponding to those of the purified MT isoforms. This technique is useful for identification of these proteins. However, it is difficult to get those pure isoforms. Commercially available MT isoforms

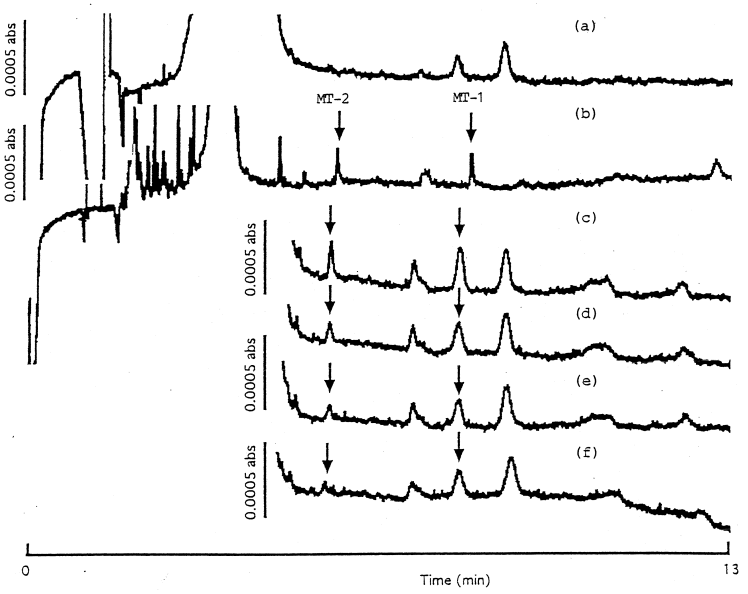

Fig. 3. CZE electropherograms of the mouse liver cytosol fraction after the addition of the anti-MT monoclonal antibody. (a) Anti-MT monoclonal antibody, (b) cytosol fraction without heat treatment, (c) cytosol fraction 2 min after the addition of the antibody, (d) cytosol fraction 15 min after the addition of the antibody, (e) cytosol fraction 30 min after the addition of antibody and (f) cytosol fraction 60 min after the addition of antibody. The specimen was loaded onto the column for 10 s by gravity and monitored at 214 nm.

have many contaminants in them as shown in the previous report [7], and are not suitable for using in this method, although the main peak of MT-1 may be detected. Therefore, we tried to identify the MT isoforms by the addition of the anti-MT antibody. Purified MT-1 was shown more reactive with the IgG of the antibody than MT-2 (Fig. 2). In addition, from the results that the peak of MT-2 in the cytosol decreased time-dependently after the addition of the antibody (Fig. 3), the peak of MT-2 in the cytosol could be detected by using the present method. In contrast, the peak of MT-1 migrated faster after the addition of the antibody and the peak was overlapped with the former small peak of the antibody and the peak area increased temporary. Although the peak area decreased time-dependently 15 min after the addition of the antibody, the former small peak of the antibody disturbed the identification of MT-1. It is necessary to observe the decrease of the peak carefully. To use purified IgG is suitable for the identification of MT isoforms, especially for the identification of the MT-1 isoform, but as described in Section 2, a high concentration of IgG is needed for one experiment. As the antibody is expensive, it is not suitable that the IgG fraction is routinely used for the identification of MT isoforms. Therefore, we recommend using it together with the two methods for the identification of MT-1 isoform in the cytosol fraction. One is to be coincided with the peak of either purified MT-1 or commercial available standard MT-1 and the other is to decrease the peak after addition of the antibody. A large peak shown in the antibody analysis was albumin.

Based on these findings, the MT-1 and MT-2 isoforms in Zn-injected mouse liver can be separated using CZE analysis at a physiologic pH and identified by the addition of the antibody.

References

[1] M.A. Strege, A.L. Lagu, J. Chromatogr. A 780 (1997) 285.
[2] Y. Xu, Anal. Chem. 67 (1995) 463R.
[3] M.P. Waalkes, in: J.A. Thomas (Ed.), Endocrine Methods, Academic Press, New York, 1996, p. 371.
[4] M. Sato, K.T. Suzuki, Biomed. Res. Trace Elem. 6 (1995) 13.
[5] P. Moffatt, F. Denizeau, Drug Metab. Rev. 29 (1997) 261.

[6] M.-C. Huang, P.K. Pan, Z.F. Zheng, N.C. Chen, J.Y. Peng, P.C. Huang, Gene 211 (1998) 49.
[7] T. Minami, H. Matsubara, M. O-higashi, N. Otaki, M. Kimura, K. Kubo, N. Okabe, Y. Okazaki, J. Chromatogr. B 685 (1996) 353.
[8] V. Virtanen, G. Bordin, A.-R. Rodriguez, J. Chromatogr. A 734 (1996) 391.
[9] T. Minami, H. Matsubara, M. O-higashi, K. Kubo, N. Okabe, Y. Okazaki, Electrophoresis 17 (1996) 1602.
[10] M. Ebadi, F. Perini, K. Mountjoy, J.S. Garvey, J. Neurochem. 66 (1996) 2121.
[11] S. Kobayashi, J. Sayato-Suzuki, Biochem. J. 251 (1988) 649.
[12] M.V.R. Kumari, M. Hiramatsu, M. Ebadi, Free Rad. Res. 29 (1998) 93.
[13] H.M. Chan, M.G. Cherian, I. Bremner, Toxicol. Appl. Pharmacol. 116 (1992) 267.
[14] L.D. Lehman, C.D. Klaassen, Anal. Biochem. 153 (1986) 305.
[15] M.P. Richards, J. Chromatogr. 482 (1989) 87.
[16] B. Berger, P.E. Hunziker, C.R. Hauer, N. Birchler, R. Dallinger, Biochem. J. 311 (1995) 951.
[17] S. Miyairi, S. Shibata, A. Naganuma, Anal. Biochem. 258 (1998) 168.
[18] J.H. Beattie, M.P. Richards, R. Self, J. Chromatogr. 632 (1993) 127.
[19] M.P. Richards, J.H. Beattie, J. Chromatogr. 648 (1993) 459.
[20] Q. Lu, S.M. Bird, R.M. Barnes, Anal. Chem. 67 (1995) 2949.
[21] H. Wittrisch, S. Conradi, E. Rohde, J. Vogt, C. Vogt, J. Chromatogr. A 781 (1997) 407.
[22] C.B. Knudsen, J.H. Beattie, J. Chromatogr. A 792 (1997) 463.