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Review

Study of metallothionein using capillary zone electrophoresis

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Abstract

Metallothioneins (MTs) have many different functions in tissues, but the roles of individual isoforms are still not entirely clear. Capillary zone electrophoresis (CZE) is a powerful method for the separation of substances because of its small sample requirement, rapid analysis, high sensitivity and high resolution. The separation and identification of mammalian MT-1, MT-2, and MT-3 and class III MTs by CZE has been reported. Uncoated and polyacrylamide-coated capillary tubes were recently used for the separation of MTs, and a UV detector is usually employed for observations of peaks of MTs. Small changes to the structure and metal components of MTs are reflected in the migration times of the peaks. N-acetylated and non-acetylated MTs can be separated and identified by CZE–mass spectrometry (MS). In addition, metal complexes with MTs can be characterized by CZE–proton-induced X-ray emission (PIXE) detector and CZE–inductively coupled plasma (ICP)–MS. For the quantification of an MT isoform, the peak area of UV absorption is used, but the technique has problems. One is lack of a purified isoform standard. The other is the need for a suitable internal standard substance. CZE–ICP–isotope dilution (ID)–MS is also reported to be able to quantify MT isoforms. CZE combined with other techniques is very effective for separation and quantitative and qualitative analyses of MT isoforms in biological materials.

Keywords: Reviews; Metallothionein; Isoform

Contents

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1. Introduction

Metallothionein (MT) is a heat-stable low molecular mass protein found in the tissues of various species of animalia, plantae, and microbes. According to their structural characteristics, MTs have been classified among three groups and mammalian MTs are included in class I MTs [1]. MT proteins are rich in cysteine, but lack disulfide bonds, aromatic amino acids and histidine. Furthermore, MT shows high-binding capacities with metals. MT is capable of binding monovalent and divalent cations and reduces metal toxicity. The structure of MT consists of two domains. Divalent cations like zinc(II) bind four atoms in the α-domain and three atoms in the β-domain. Monovalent cations like copper(I) bind six atoms in each domain [2]. In this way, all sulfhydryl groups in cysteine residues are used up to bind metals. MT has four major isoforms; MT-1 and MT-2 are acute phase proteins, while MT-3 and MT-4 are not.

MT-1 and MT-2 are expressed together in same certain in response to various stimulators such as metals, glucocorticoid, oxidative stress, cytokines, and so on. Certain metals bound both to MT-1 and to MT-2 can be easily exchanged for higher affinity metals. The functions of both MTs are divided broadly into four categories: (1) reducing metal toxicity, (2) maintenance of the homeostasis of essential metals, (3) scavenging of free radicals, (4) cell growth and proliferation [1,2]. Nevertheless, the difference in function between the two MTs is still unclear, although it has been stated that MT-1 mainly acts in metal metabolism and MT-2 in cell growth.

The MT-3 isoform was isolated from human brain and originally named growth inhibitory factor. Meanwhile, MT-4 was discovered in squamous epithelial cells. It was thought that neither isoform was an acute phase protein, but recently MT-3 was found to be induced by several stimuli in the brain. Also the level of MT-3 in the brain changes in patients with neurodisease [3,4]. Furthermore, immunohistochemically, both isoforms are stained in various organs. However, the study of MT-3 and MT-4 isoforms is yet to begin.

The study of MT in biological materials has employed the following methods: (1) quantification of MT in cells or tissues, (2) defense effects of MT after pre-induction of MT, (3) locality of induced-MT using immunostaining or immunoelectron microstaining, (4) detection of MT mRNA by RT-PCR, (5) experiments using MT knock-out or over-expression in mice, and (6) changes of MT protein structure.

There are several reviews for quantification [5,6], and Dabrio et al. [7] and Połeć et al. [8] recently reviewed MT quantification and hyphenated techniques. Therefore, the present review gives only an outline about general quantification and hyphenated techniques. Capillary zone electrophoresis (CZE) has now taken the place of HPLC. However, proteins often adsorb to the inner wall of uncoated capillary tubes. Such unfavorable adsorption can be interrupted by modification of the wall by coating with neutral polymers like liner polyacrylamide or neutralizing of charges on the wall with ionic additives of countersign like polyamines. The aim of the present review is to introduce several techniques for examining the properties of MT isoforms using CZE, and to show the future study of MT using CZE. The term MT in the present review refers to both the MT-1 and MT-2 isoforms unless stated otherwise.

2. General quantification and hyphenated techniques for MT

2.1. Quantification methods for MT

The methods used to quantify the total amount of MT in biological materials are divided into two types according to specificity. One is based on the measurement of the amount of metal bound to MT. As metals bind proportionally to the amount of MT, metal contents indicate the MT content. The affinity to MT differs among metals (Hg(II)>Ag(I)~Cu(I)> Cd(II)>Zn(II)) [2]. The MT produced in response to various stimuli is composed mostly of Zn, though higher affinity metals form different metal-thioneins. Therefore, the simplest method for the quantification of MT is the Cd-hem method [9]. The Cd-hem method utilizes two properties; that MT is a heat-stable protein and that seven atoms of Cd instead of Zn bind one molar of MT protein when excess Cd is added to cytosol fraction. Hg- and Ag-saturation methods apply the same theory. The other method of quantifying the total amount of MT is based on the assay of protein content. The quantity of sulfhydryl residues can be used to measure MT protein content, while a radioimmunoassay (RIA) and enzyme-linked immunosorbent assay (ELISA) for MT protein have also been developed. Five of these methods for measuring MT; the Cd-hem method, Hg-saturation assay, RIA, thiolate group determination, and Sephadex G-75/atomic absorption spectrometry (AAS) were compared based on recovery rates using standard MT [10]. A good rate was obtained using the RIA and Cd-hem methods (97 ± 12 and 105 ± 10%, respectively), whereas the recovery rates for Hg-saturation assay, thiolate group determination, and Sephadex G-75/AAS were not particularly high. In addition, ELISA and RIA show high sensitivity for determining MT levels in tissues, while the Cd-hem method covers a wide range. Electroanalytical techniques are also used to quantify MT [11]

2.2. Separation methods and hyphenated techniques

If heat treatment is not desired for the quantification, or if the properties of each isoform are to be examined, a separation technique is used before the detection. For separation, Sephadex G-75 gel permeation chromatography (GPC), anion-exchange chromatography, high-performance liquid chromatography (HPLC), and CZE are often used, while UV absorption, fluorescence detection, AAS, mass spectrometry (MS), and inductively coupled plasma (ICP)–MS are used for detection of MT. MT isoforms and MT content are assayed by using a combination of separation and detection techniques. Until recently, GPC–AAS, HPLC–AAS, and HPLC–UV were often used to identify the metal species binding MT and also for the quantification of the MT content of tissues. Using these techniques, the existence of not only MT isoforms but also MT dimer was established [12]. Recently, MS-combined techniques have been reported [8]. Numerous isoforms and sub-isoforms have been identified using HPLC–MS [13,14]. In addition, an oxidized form of MT was quantified by ICP–MS [15]. An ELISA can measure each MT isoform [16,17].

The mRNA levels of each isoform in tissue have been assayed by RT-PCR [7,18]. The human MT gene family has 17 genes (13 genes for MT-1, two for MT-2, one for MT-3, and one for MT-4) on chromosome 16 [1]. The change in gene expression depends on the organ and disease [19–21]. Why does the MT gene family have so many genes? Does the expression of each gene reflect an increase in the protein level of each MT isoform? Are there any differences in function between the isoforms? To answer these questions, it is necessary to study the expression of each gene and the function of each isoform. In addition, when examining the properties of an isoform, either HPLC or CZE is used to separate MT isoforms from biological specimens because the procedure is quick and requires little sample.

3. Capillary zone electrophoresis

A separation technique using CZE was developed in the 1990s and has been used in the study of MT since 1993 [22]. Early studies using CZE for MT analysis were to develop the most suitable conditions for the separation of MT. To this end, capillary tubes, electrolytes, instruments, detectors, and so on were examined [23–25]. Strategies for the study of

MT using CZE were reviewed in 1998 [26]. To date, uncoated and polyacrylamide-coated capillaries have mainly been used for the analysis, and a previous review [26] recommended the use of borate–SDS electrolytes at an alkaline pH in uncoated capillaries. However, we found that Tris–HEPES buffer without detergents at a neutral pH gave the best resolution in polyacrylamide-coated capillaries [27]. Most promising techniques since the review was presented in 1998 may be the identification of each MT isoform and elements bound to MTs using proton-induced X-ray emission (PIXE), MS and ICP–MS after separation. However, as high resolution and rapid analysis are required for the separation of closely related isoforms, CZE using a UV detector is still suitable for this purpose.

3.1. Comparison of CZE with other separation techniques

When the fraction, in which zinc was detected, of the cytosol of zinc-treated sheep effluent from Sephadex G-75 was subjected to CZE with an uncoated capillary, very clear peaks of MT-1 and MT-2 were obtained and no further chromatographic analysis was needed [22]. When the separation of purified MT was achieved by uncoated-capillary CZE and by reversed-phase (RP) HPLC, using a C_8 column eluted with a linear acetonitrile gradient, many more peaks could be detected by CZE than HPLC [28]. While RP-HPLC separates proteins based on differences in hydrophobicity, CZE utilizes a combination of differences in mass and charge. Therefore, CZE is effective for the separation of MT analogues.

3.2. Protein adsorption to capillary inner walls

Proteins easily adsorb to the inner walls of capillary tubes when the silanol groups of the walls are ionized to a pH of 3 or more. In CZE with small amounts of protein as sample, this kind of adsorption is fatal. For example, 50% or more of the myoglobin fluorescent marker is adsorbed at pH 5.5–7.0 [29]. Under these conditions, a high resolution that reflects subtle differences in the charge is not possible for proteins.

For the CZE of MT isoforms in uncoated capillaries, buffer solutions with additives such as sodium dodecyl sulfate (SDS) [30,31] or methanol [32] have been used to obtain high resolution. These additives have the effect not only of reducing protein–wall interaction, but also of interacting to MTs. Improvement in the separation or resolution of MT isoforms from chicken liver extract was achieved by increasing the SDS concentration contained in 100 m*M* sodium borate buffer pH 8.4, although the migration times of each peak were longer [26]. A similar effect was achieved by using a 150–150 m*M* Tris–tricine buffer system (pH 7.75) containing methanol (30–40%, v/v) [32]. The other technique to reduce protein–wall interaction using uncoated capillaries is to analyze at low pH. However, MTs are unstable at pH 2.5 as metals dissociate from the MTs and metal-free proteins, apothioneins, are detected.

3.3. Merely a coating, but a coating nevertheless

The choice of conditions such as the type of capillary, buffer concentration, buffer composition, pH, temperature, and voltage decide the resolution of the MT isoforms to be separated by CZE. Uncoated capillaries offer advantages in terms of analysis time and capillary lifespan, whereas surface-modified capillaries enhance the resolution of MT isoforms.

3.3.1. Coating of the capillary inner wall

The purpose of modifying the inner wall of the capillary tube is suppression of protein adsorption and restriction or reversal of osmotic flow. A modification of the capillary inner wall to suppress protein adsorption necessarily suppresses osmotic flow. Details about coatings are described in recent issues of *Electrophoresis* [33,34].

In the separation of MT isoforms, polyacrylamide-, polyamine-, and sulfonic acid-coated capillaries have been used [27,35–38]. The polyacrylamide coating procedure used in our studies is illustrated in Fig. 1. MT specimens migrated at a pH near neutral or slightly higher when applied to these coated capillaries. CZE with a linear polyacrylamide-coated capillary is carried out in the absence of such additives as dynamic coating agents.

3.3.2. Zone electrophoresis in a polyacrylamide-coated capillary

When a commercially available MT-1 standard prepared from rabbit liver was loaded onto uncoated

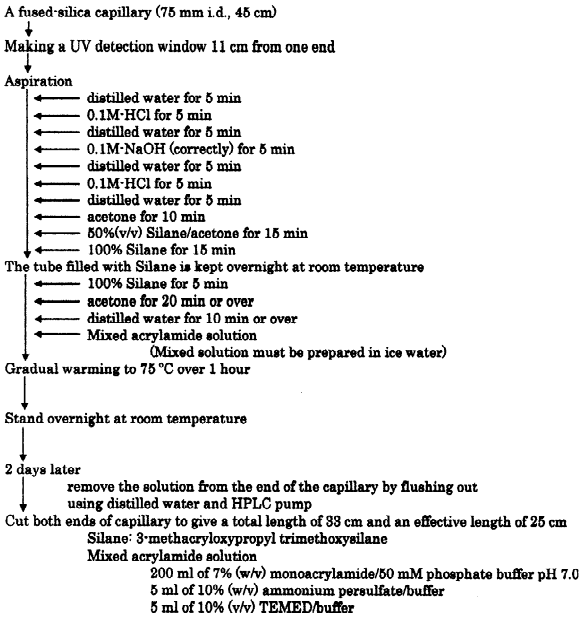

Fig. 1. Preparation of polyacrylamide-coated capillary.

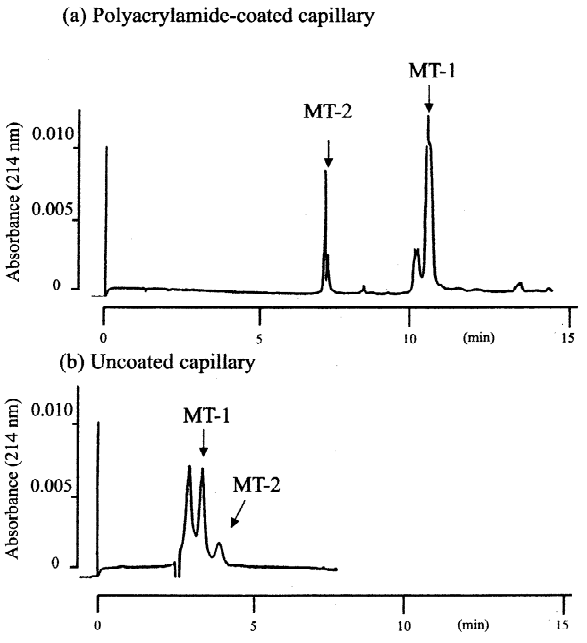

Fig. 2. Electropherograms of commercially available MT-1 isoforms using polyacrylamide-coated and uncoated capillaries. Commercially available standard MT-1 (0.1 mg/ml, rabbit liver, Sigma) was loaded onto the column for 5 s by gravity. HEPES–Tris buffer (50 m*M*, pH 7.4) served as the running buffer. The analysis was performed at 25 kV and 26±1 °C and monitored at 214 nm. Reproduced with permission from [46].

and polyacrylamide-coated capillaries, and electrophoresis was performed at 25 kV, and 26±1 °C using 50 m*M* HEPES–Tris buffer (pH 7.4), and monitored at 214 nm, the specimen showed ten or more peaks on the coated capillary, but only three wide peaks on the uncoated capillary (Fig. 2) [39]. Both MT-1 and MT-2 were detected within 15 min on the polyacrylamide-coated capillary. As the inlet was the cathode, MT-2 migrated faster than MT-1 under conditions of no osmotic flow on the coated capillary. These results show that CZE in a polyacrylamide-coated capillary tube is an efficient measure of the separation of MTs with many isoforms because it can be done in a buffer solution of near physiological pH that would be expected to reflect subtle differences in the charge of the amino acid side-chain ionizable for proteins.

4. Study of MT using CZE

The study of MT using CZE can be divided into the following steps: (1) estimating the properties of each isoform, and (2) quantifying the isoforms. Recently, sub-isoforms and non-acetylated isoforms were found using CZE–MS [40,41], and elements combined with MT isoforms were detected by CZE–ICP–MS [4] and CZE–PIXE [42].

4.1. Identification of MT isoforms by CZE

4.1.1. Sample preparation

A very small volume of sample (less than 100 nl) is subjected to CZE, and the analysis is performed quickly (within 15 min). Therefore, as described in the previous review [26], it is important to prepare stabilized, concentrated, and relatively contaminant-free samples for loading onto the capillary. When a cytosol fraction prepared from 10% homogenate is directly subjected to CZE, many peaks are detected on the electropherogram, and some may co-migrate with MT isoforms [43]. Given that MT-1 and MT-2 isoforms containing zinc and/or cadmium are heat-stable, applying the supernatant of the cytosol after heat treatment is a simple technique [44]. The majority of proteins other than MTs can be removed from the cytosol fraction by heat treatment within 2 min and standard MT-1 and MT-2 are stable to the treatment. Conversely, MTs containing copper are

not stable against heat treatment. Usage of either a molecular filtration unit or solid-phase extraction is a better technique for enhancing the selectivity and sensitivity [45]. Furthermore, after partial purification, stored MTs are not stable on CZE, even if stored at −80 °C [46,47].

4.1.2. Detection methods

Detection methods for separated MT isoforms include UV absorption, PIXE, and MS.

UV absorption is often used after separation because of its convenience. From its high intensity, absorbance of 200 or 214 nm is generally used for detection of peaks in CZE. In addition, a method in which migration times are compared with those of purified isoforms is generally employed [27]. However, the matrix components in the specimen, the viscosity, and the life span of the capillary affect the migration time, even if no contaminants co-migrate with the isoforms.

As the metal–thiolate bonds in MTs exhibit distinct absorption spectra, it is possible to obtain spectral information concerning all separated components by monitoring simultaneously at different wavelengths such as 200, 214, 254, and 280 nm [26,30]. For example, apothionein shows low absorbance at 220 nm and above. The Zn–S bond shows a low-energy charge-transfer transition band at 231 nm, the Cd–S bond at 250 nm, and aromatic amino acids at 280 nm. Therefore, it is possible to speculate from the absorbance at 214 and 280 nm whether the peak is an MT isoform or not.

A more direct method is to add anti-MT monoclonal antibody to the specimen [48]. When MT-1 and MT-2 were mixed with anti-MT antibody, and subjected to CZE, the peaks of MT-1 and MT-2 decreased in height compared to those without the antibody dependent on time, enabling us to identify then. However, as the anti-MT antibody is expensive, it is impractical to regularly use large volumes for identification. We, therefore, developed a rapid and simple method to detect and identify MT isoforms with high efficiency. Incidentally, as the peaks of MT isoforms decreased on CZE after the addition of excess Cd to the specimen [49], EDTA was added at different concentrations, and CZE performed [50]. Within a narrow range of EDTA concentrations, the MT peaks were recovered, while beyond this range, they disappeared again. Thus, the two peaks were confirmed as MT-1 and MT-2 based on migration times that corresponded with those of the purified MT isoforms, and the decay of their peaks following the addition of EDTA, which depended on incubation time and concentration added, as shown in Fig. 3. The unfolding of metal–mercaptide transitions and/or polymerized adducts may decrease the absorption of UV after EDTA, although the mechanism is not clear. Therefore, both isoforms can be identified by CZE with or without the addition of EDTA. Similarly, for the several minor peaks that are expected to be MT isoforms, a decay of the peaks was observed. As both N-acetylated and non-acetylated forms were observed on CZE–MS [41] and HPLC–ICP–MS [51], and as the minor peaks also migrated dependent on pH, those minor peaks may have been MT sub-isoforms.

PIXE detector is metal-specific as well as fist-

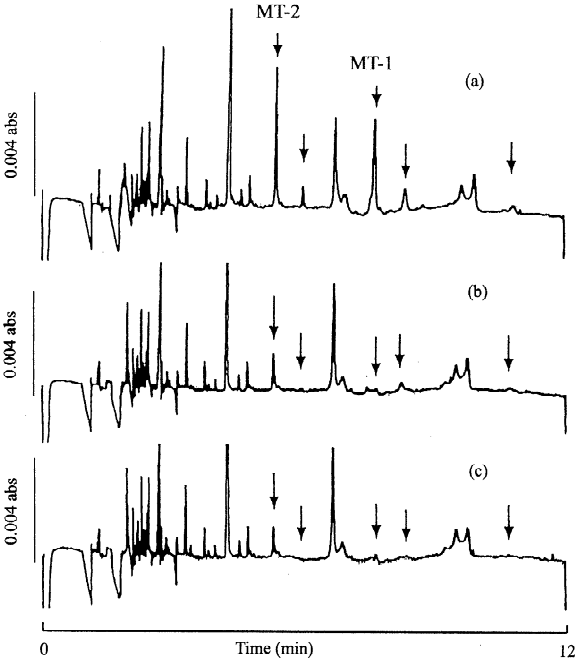

Fig. 3. Effect of EDTA on heat-treated cytosol fraction of Zn-injected mouse liver. (a) Heat-treated cytosol fraction. The specimen was incubated with EDTA for 2 (b) and 15 min (c). The specimen was loaded onto the polyacrylamide-coated column for 10 s by gravity and monitored at 214 nm. HEPES–Tris buffer (50 m*M*, pH 7.4) served as the running buffer. The analysis was performed at 20 kV and 26±1 °C. Reproduced with permission from [50].

sensitive and of great use for the characterization of MT isoforms separated by CZE [42]. Neither the separation conditions nor the buffer composition interfered with the generation of the X-ray signals. By using a PIXE, metals bound to MT isoforms can be identified easily and with accuracy. In addition, it was reported that PIXE had lower detection limits than UV in some cases [42].

As described above, MS is the most suitable method for the identification of separated MT isoforms by CZE. While CZE–MS can provide specific molecular information, CZE–ICP-sector field MS can obtain the simultaneous information on the elements bound with MT isoforms as ICP–MS can detect elemental intensity. Recently, MT-3 as well as MT-1 and MT-2 were detected in the cytosol fraction of human brain using CZE–ICP–MS [4,8]. Levels of MT isoforms can be quantified by CZE–ICP-sector field MS using the sulfur isotope dilution method [4]. Measuring the amounts of elements such as sulfur, zinc, cadmium, and copper in MT isoforms simultaneously allows for the characterization of the metal–thionein complex in MT isoforms. Furthermore, by adding an isotope like ^{63}Cu to MT samples, all isoforms can be detected and quantified. In human brain, MT-1 and MT-3 levels in temporal and occipital regions were reported to be lower in patients with Alzheimer's disease than in control subjects using CZE–ICP–MS. The detection limit of CZE–ICP–MS for MT-1, MT-2, and MT-3 based on ^{64}Zn was 2.02, 5.45, and 7.6 μg/ml, respectively, and the MT-1 and MT-2 concentration based on ^{114}Cd was 0.011 and 0.023 μg/ml, respectively [4].

4.2. Properties of MT isoforms on CZE

As the peak migrated at different times even if the pH was changed slightly from 7.60 to 7.70 as shown in Fig. 4, the migration is affected even when one amino acid of the protein component is modified, and when the metal component is different. The change in the ratio of MT-1 to MT-2 in tissues after induction is easily observed by CZE. In addition, effects of substances that interact with MTs directly, like metals, chelators, and free radicals, are also observed by changes in migration times and peak areas. Furthermore, when the peaks of sub-isoforms and non-acetylated isoforms are identified by UV

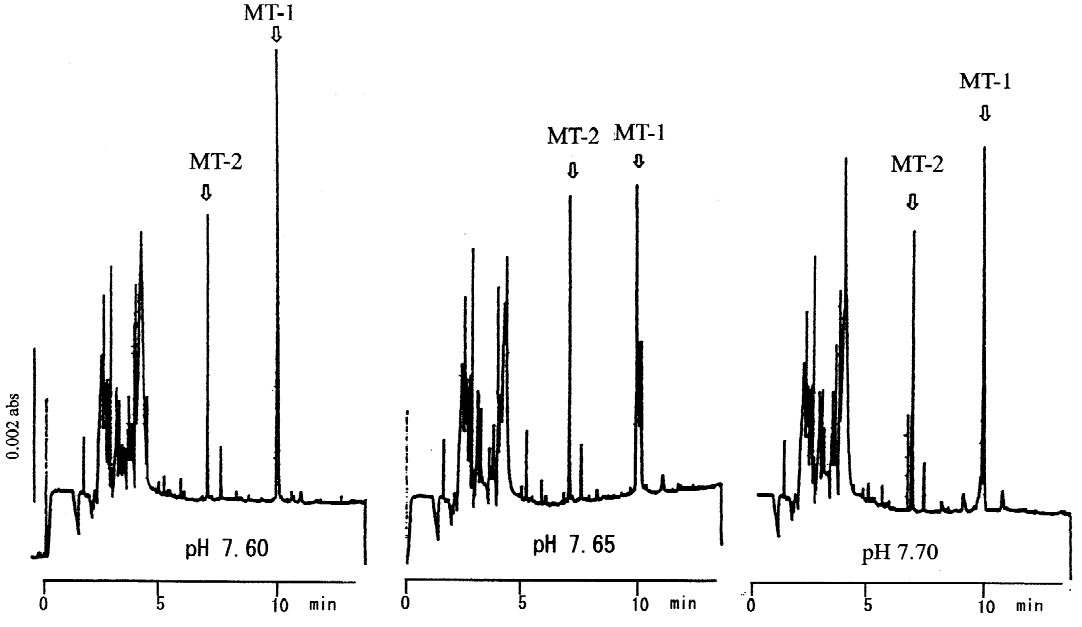

Fig. 4. Effect of pH on the separation of cytosol fraction of Zn-injected mouse pancreas. Heat-treated cytosol fraction was loaded onto the polyacrylamide-coated column for 5 s by gravity and monitored at 214 nm. HEPES–Tris buffer (50 m*M*, pH 7.4) served as the running buffer. The analysis was performed at 20 kV and 26±1 °C. Reproduced with permission from [46].

detector following the peaks are pre-identified by MS and other techniques, the function of each MT isoform may be elucidated further.

As described above, metal components and ratios in MTs are also determined using PIXE and ICP–MS detector. Further investigation may begin in this area.

The analysis of class III MTs like phytochelatins as well as MT isoforms has been developing based on CZE analysis [52]. Phytochelatins [$(\gamma$-Glu–Cys$)_n$–Gly, $n \geq 2$] are produced in plants in response to heavy metals, and the separated peaks of GSH, γ-glutamylcysteine, and phytochelatins ($n=2$–9) can be observed by CZE.

4.3. Quantification of MT isoforms by CZE

The detection limits and linear ranges of various methods are summarized in a recent review [7]. For CZE analysis in the quantification of MT, the peak area on the UV detector is usually used. We quantified MT-1 and MT-2 contents by CZE using a polyacrylamide-coated capillary at a neutral pH without any detergents, and obtained a good linear correlation between the concentration of MT isoforms and the peak areas from 5 to 100 μg/ml [53]. Conversely, however, there are two major problems with the quantification of MT isoforms in biological materials using a UV detector for CZE. One is the normalization of MT isoforms. Commercially available standards of MT isoforms are not adequate for use in CZE; for example, the MT-1 standard produced many peaks including MT-2 on CZE. Even if the standards are quantified by other methods like ELISA, the data can not be used to evaluate the content of the peaks observed by CZE. It is usually difficult to prepare standards that show a single peak because CZE has high-resolution efficiency. The other problem is how to maintain constant analytical conditions. Due to differences in the viscosities of specimens and in life span of capillaries, an internal standard is required for CZE. We selected a carbonic anhydrase as internal standard. Since heat treatment, molecular filtration or solid-phase extraction must be performed before the analysis, it is normal to add internal standard to specimens after such treatments, but it is desirable to add the standard to the homogenizing medium prior to homogenization.

The ICP–isotope dilution (ID) MS method combined with CZE is used for the quantification of MT isoforms [4]. The principle behind the method is that concentrated sulfur isotope mixtures on-line with substances separated by CZE and the ratio of $^{32}S/^{34}S$ are calculated. Using a molar ratio of 21 sulfur atoms per molecule, the quantities of the MT isoforms are calculated. In addition, in combination with the detection of metals in peaks, peaks of each MT like MT-1, MT-2, and MT-3 can be detected and concentrations can be calculated without a standard curve. A constant sample flow-rate into plasma for ICP–MS and a constant mixture of diluted isotope are needed for quantification.

5. Conclusion

MT in mammals has four major isoforms, and the MT gene in humans is located on chromosome 16 with at least ten genes being functional [1]. The roles of individual MT isoforms in tissues are not well known; especially the biological and physiological functions. Recently, the existence of different forms of MT, i.e. sub-isoforms, non-acetylated products, metal complexes, and a dimer have been reported using CZE with UV and other detectors. CZE in a coated capillary, which is a powerful technique for the separation of protein isoforms and has gained general acceptance, appears to give a resolution that is higher than that of CZE in a bare capillary. Many isoforms, MT-1, MT-2, MT-3, and class III MTs in plants have been detected by CZE. MT-3 and MT-4 in various kinds of organs will be found if CZE is performed in either a coated or a bare capillary in the near future. The peaks of MTs separated by CZE can be easily identified based on migration times, if any purified MTs prove an effective marker for UV absorption. Therefore, the use of PIXE, MS, and ICP–MS in combination with CZE is expected as a detection technique to discover unknown MT components within peaks separated by CZE. By applying the above techniques, the MT peaks detected on UV absorption may be realized in more detail. Further information on the characteristics and quantities of individual isoforms will be revealed in the near future.

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