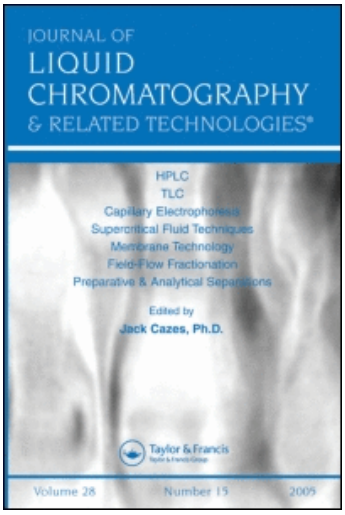

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APPLICATION OF CAPILLARY ZONE ELECTROPHORESIS TO THE SEPARATION OF METALLOTHIONEIN ISOFORMS

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ABSTRACT

Metallothioneins (MTs) from various eukaryotic species were subjected to capillary zone electrophoresis (CZE) performed under the following conditions: Capillary: untreated polyimide-clad fused silica 75 μm I.D. x 94 cm; Loading and Running Voltage: 30 kV; Electrode Buffer: 50 mM Tris-HCl pH 9.1; Sample Buffer: 10 mM Tris-HCl pH 9.1; Sample Volume: 250 μl; Detection: UV absorbance at 214 nm; Loading Method: electrokinetic migration; Loading Times: 1-6 seconds. Complete separation of MT-1 and MT-2 isoforms was achieved in less than 6 min. Using a rabbit liver Cd,Zn-MT standard (0.5 mg/ml), a linear relationship was found to exist between the voltage used to load the MT sample and the integrated peak area of the individual MT isoforms. Similarly, the integrated peak area of the separated MT isoforms was a linear function of the time used to load the sample. These findings suggest that with appropriate standardization CZE is capable of both qualitative and quantitative determinations of MT isoforms. In CZE, the concentration of MT in solution determines the limit

of sensitivity. MT could be accurately detected at a concentration as low as 10 μg/ml. CZE was applied to assess thermal and proteolytic degradation of rabbit liver MT. CZE represents a rapid analytical technique for the characterization of MT isoforms that requires very little sample yet provides excellent resolution of individual MT isoforms.

INTRODUCTION

Metallothioneins (MTs) are low molecular weight, heavy-metal-binding proteins characterized by high levels of cysteine residues (30 mole%). MTs are unique in that the metals that bind to the protein also induce its synthesis. Moreover, in many eukaryotic species, a family of MT genes give rise to two major classes of MT isoforms (MT-1 and MT-2) which differ in their net charge resulting from specific amino acid substitutions. Because of its unique structure, its metal-binding properties and its suggested role in the metabolism of heavy metals, MT has been the focus of much investigation (1). As a result, an ongoing need exists for new and more sensitive analytical techniques applicable to the separation and quantification of MTs from a variety of tissue and extracellular fluid matrices.

Early chromatographic-based isolation techniques were indirect and relied on the quantification of metals bound to the protein (2). Newer techniques involving direct detection of the thionein peptide have gained wider application. Immunological assays (RIA and ELISA) offer the highest level of detection sensitivity for MT and are particularly useful for analyzing MT present in low quantities such as in urine or plasma (3-5). However, most immunoassays lack the ability to separately quantify individual MT isoforms.

Reversed phase high performance liquid chromatography (RPHPLC) offers reasonably high detection sensitivity (ca. 150 picomoles) combined with the ability to resolve individual MT isoforms, but requires the use of organic solvents and costly columns (6-8). Recently, CZE has been used in the analysis of proteins and peptides (9-10). CZE, employs a narrow bore capillary tube and high voltage to separate multicharged species such as proteins and peptides in an applied electric field based on their charge-to-mass ratio at a given pH. The advantages of CZE over other separation techniques include: 1) rapid analysis times (< 30 min.), 2) small sample size requirements (< 50 nl), 3) nanogram sensitivity, 4) high resolution and 5) improved selectivity. The purpose of our study was to determine the applicability of CZE to the separation of MT isoforms.

MATERIALS AND METHODS

Instrument:

The CE instrument consisted of a CV^4 variable UV/VIS detector (ISCO Inc., Lincoln, NE) fitted with a capillary cell holder and an external capillary holder which also housed the anode and cathode and the electrode buffer containers. A Brandenburg Ltd. Alpha III power supply was used to apply voltage across the capillary. The capillary used was an untreated, polyimide-coated, fused silica tube (75 μm I.D. x 94 cm). Data were collected using a personal computer and analyzed by the LabTech Acquire and LabTech Chrom software programs (Laboratory Technologies Corp., Wilmington, MA).

Materials:

Standard MTs were either purchased from a commercial source (rabbit liver and horse kidney Cd,Zn-MTs, Sigma Chemical Co., St. Louis, MO) or

were prepared from Zn-induced tissue by sequential gel permeation and ion exchange column chromatography (2). Chick liver Zn-MT was further purified by reversed phase HPLC according to the method of Richards and Steele (11) prior to CZE.

Methods:

CZE was performed as follows: Prior to a series of runs, the capillary was flushed with 0.1M KOH, followed by water and finally running buffer (50 mM Tris-HCl, pH 9.1). With the capillary filled with running buffer, a voltage of 30 kV was applied and the system allowed to stabilize for 1 hr. prior to the initiation of separations. Samples of MT were dissolved in 10 mM Tris-HCl, pH 9.1 and were loaded into the capillary using electrokinetic migration at the specified voltage and loading time. After a sample was loaded, the run was initiated by applying 30 kV across the capillary. MT isoforms were detected by their absorbance at 214 nm. Thermal stability of MT was assessed on samples of rabbit liver MT stored either in the refrigerator at 4°C or on the bench top at room temperature (22°C) for a period of time in excess of one month. Protease (subtilisin) digestion of MT was carried out essentially according to the technique of Winge and Miklossy (12) except that the MT samples were incubated in the presence or absence of 1 mM EDTA for 1.5 hr. at room temperature prior to the addition of subtilisin. The samples (with or without EDTA) were monitored by CZE at 0, 8 and 16 hr. after the addition of subtilisin.

RESULTS AND DISCUSSION

The major isoforms (MT-1 and MT-2) for different eukaryotic MTs were resolved in less than 6 minutes when subjected to CZE in Tris buffer at

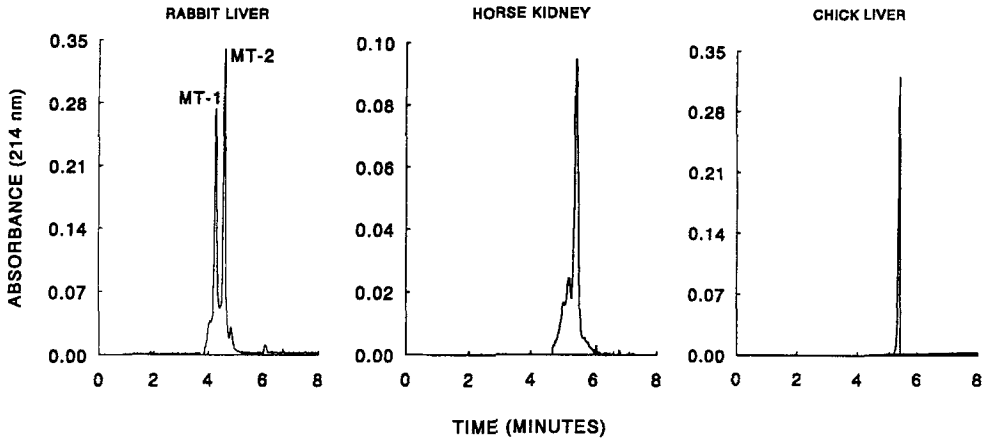

Figure 1. Capillary zone electropherograms of MTs purified from rabbit liver, horse kidney and chick liver. All MTs were dissolved in 10 mM Tris-HCl pH 9.1 at a concentration of 1.0 mg/ml, loaded for 2-4 sec. by electrokinetic migration and run at 30 kV in 50 mM Tris-HCl, pH 9.1.

alkaline pH (Figures 1 & 2). When corrected for differences in electroosmotic flow, it was found that the major MT isoforms from different species comigrated (data not shown). That is the MT-1 and MT-2 isoforms each migrated as a singular group. One exception was horse kidney Cd,Zn-MT which, at pH 9.1, yielded a single predominant peak (Figure 1). When mixed with rabbit liver MT, the horse kidney MT peak comigrated with the MT-1 isoform (data not shown). RPHPLC does resolve horse kidney MT-1 and MT-2 isoforms (8,11). The reason for the incomplete resolution of MT isoforms from horse kidney by CZE is unclear at present but may be related to the conditions under which CZE was performed or to differences in the amino acid composition unique to equine MT isoforms and the resulting net charge of the MT-1 vs. the MT-2 isoform at pH 9.1. Chicken MT has been previously reported to consist of

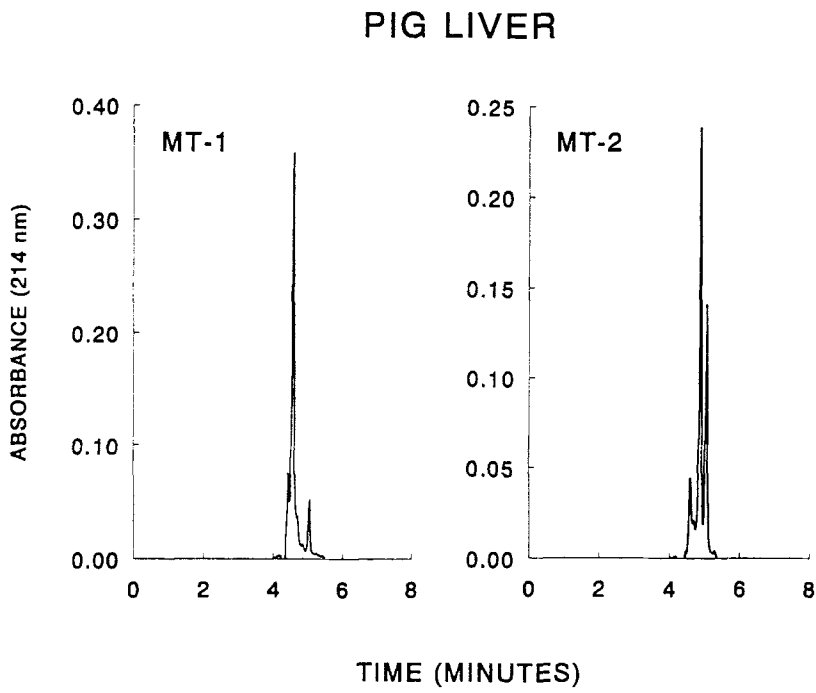

Figure 2. Capillary zone electropherograms of the major MT isoforms (MT-1 and MT-2) from Zn-induced pig (A) and sheep (B) liver previously isolated by a combination of gel filtration and ion exchange chromatography prior to subjection to CZE. Conditions for CZE were the same as those described in the legend to Figure 1.

a single isoform encoded by a single gene (13). Consistent with this view, when subjected to CZE, chicken Zn-MT yielded a single peak at pH 9.1. When MT isoforms purified by ion exchange chromatography prior to analysis were subjected to CZE, additional species could be detected for pig Zn-MT, especially for the MT-2 isoform (Figure 2A). Sheep Zn-MT isoforms (MT-1 and MT-2), on the other hand, demonstrated no significant microheterogeneity on CZE (Figure 2B) despite the fact that

B)

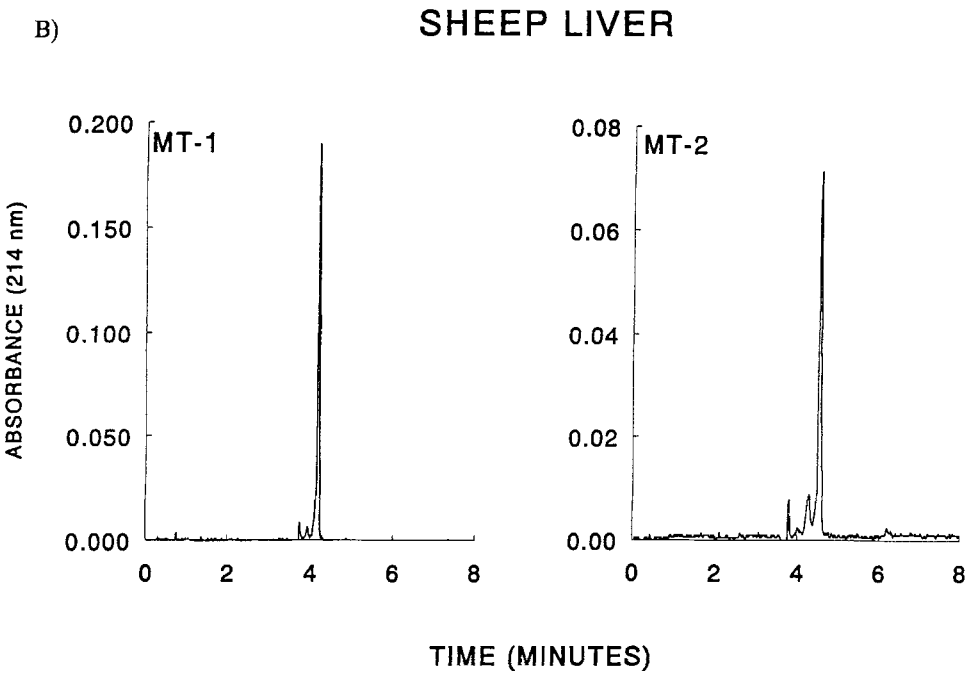

Figure 2 (continued)

genetic evidence has confirmed the existence of 4 expressed MT genes including 3 MT-1 genes and 1 MT-2 gene (14,15). It is possible that the levels of individual isoforms vary greatly and that lesser abundant species could escape detection. It is also possible that because of the type of amino acid substitutions additional isoforms would not differ with respect to charge and therefore would not be resolved by CZE at pH 9.1. Clearly, more investigation is required to distinguish among these possibilities. The occurrence of additional isoforms has also been recognized for human MT-1 which is comprised of 5 subforms (16,17). In this case, reversed-phase HPLC (RPHPLC) has been the most useful method for

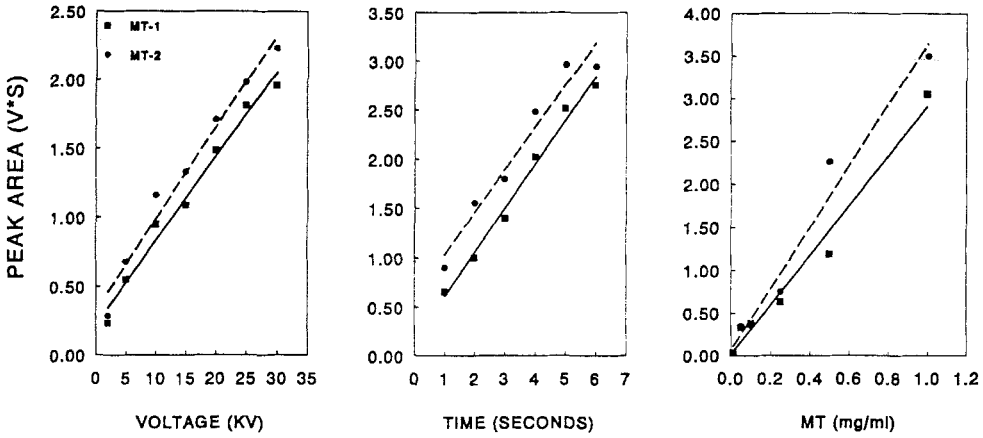

Figure 3. Integrated peak areas of the MT-1 (dashed line) and MT-2 (solid line) isoforms from a rabbit liver MT sample (0.5 mg/ml) plotted as a function of the voltage used to load the sample electrokinetically (left) and as a function of the time of loading (center). A loading time of 4 seconds was constant while the voltage was varied and a voltage of 30 kV used when the time of loading was varied. Also shown (right) are integrated peak areas of MT-1 and MT-2 plotted as a function of the total MT concentration (0.01-1.00 mg MT/ml). The running conditions were identical to those described in the legend for Figure 1.

characterizing such microheterogeneity within the major MT isoform groups (17). Because of differences in selectivity compared to RPHPLC, we believe that CZE represents a potentially useful alternative analytical tool with which to further characterize MT isoform heterogeneity.

Integrated peak area of individual rabbit liver Cd,Zn-MT isoforms was found to be a linear function of the voltage and the time used to load (by electrokinetic migration) the sample into the capillary (Figure 3). Integrated peak area was also found to be linearly related to the concentration of MT in the sample (Figure 3). This suggests that CZE could be readily adapted to quantitative MT analysis with appropriate

standardization. The concentration of MT in the sample, not the absolute amount loaded, is the major factor which determines the sensitivity of this technique as applied to the quantification of MT. In contrast, RPHPLC has the ability to concentrate analytes from dilute samples by loading a larger volume onto the column. The volume of sample that can be loaded into a standard capillary is limited (generally < 50 nl) and therefore, CZE is largely dependent on the sample analyte concentration. The observed limit of detection for our instrument was between 1 and 10 µg MT/ml when the MT was loaded by electrokinetic migration at 30 kV for 4 seconds.

Despite the fact that the capillary was neither rinsed with fresh running buffer nor regenerated with base between runs, reproducibility was, in general, good (RSDs with one exception were <10%) for migration time, integrated peak area and peak height of the major MT isoforms (MT-1 and MT-2) as well as of the ratio of the two isoforms (Figure 4, Table 1).

Thermal stability of rabbit liver Cd,Zn-MT in solution was assessed with CZE (Figure 5). The MT-1 isoform appeared to be unstable when stored at room temperature and generated decomposition products which were identified by CZE. This was apparently not the case for the MT-2 isoform stored at 22°C as no decline in peak height or area was detected. It has previously been reported that under specific experimental conditions the rat hepatic MT-1 isoform is more labile than the MT-2 isoform (18,19). In this study, both isoforms appeared to be stable when stored at 4°C in Tris buffer at pH 9.1 for an extended period of time. Based on these findings, we propose CZE as an excellent method for the evaluation of the quality and purity of MT standard solutions to be utilized in other analytical techniques such as RIA or HPLC.

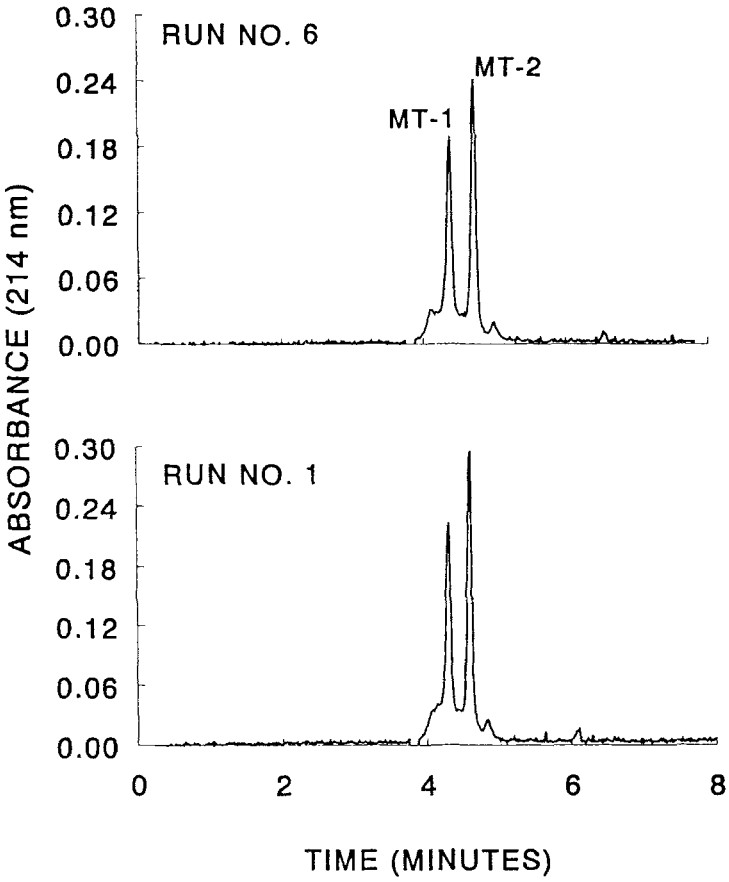

Figure 4. Reproducibility of rabbit liver MT isoform separation by CZE according to the conditions described for Figure 1. The first and sixth consecutive runs are depicted.

The proteolytic degradation of rabbit liver Cd,Zn-MT-1 by subtilisin in the presence or absence of EDTA was monitored with CZE (Figure 6). Addition of EDTA which removes Zn from the β domain of MT (12) markedly reduced the absorbance of MT-1 at 214 nm. EDTA also altered the electropherogram by introducing additional peaks. Some of these

TABLE 1

Reproducibility of Rabbit Liver Cd,Zn-MT Isoform Migration Time, Peak Area and Peak Height for Six Consecutive Runs[a]

Isoform	Time (min)	Area (V*S)	Height (V)
MT-1	Mean= 4.44 SD= 0.10 RSD= 2.21%	Mean= 1.31 SD= 0.15 RSD= 11.21%	Mean= 0.20 SD= 0.01 RSD= 5.96%
MT-2	Mean= 4.80 SD= 0.16 RSD= 3.33%	Mean= 1.44 SD= 0.10 RSD= 6.69%	Mean= 0.26 SD= 0.02 RSD= 7.81%
MT-1/MT-2[b]	Mean= 0.91 SD= 0.02 RSD= 2.20%	Mean= 0.91 SD= 0.06 RSD= 6.59%	Mean= 0.77 SD= 0.02 RSD= 3.06%

[a] SD= standard deviation of the mean, RSD= relative standard deviation.

[b] Values for the ratio of the MT-1 and MT-2 isoforms.

peaks may arise from the partial removal of Zn ions from MT-1 by EDTA. Since bound metal is an important determinant of the tertiary structure of MTs as well as the net charge, the occurrence of additional peaks on CZE may reflect alterations in either or both of these parameters. Subtilisin did apparently degrade MT-1 as judged by a reduction in peak height. Also additional more negatively charged peaks migrating more slowly than the intact MT-1 peak were detected in the electropherograms and their levels appeared to increase over time. Although Winge and Miklossy (12) found that prior EDTA treatment was necessary to partially remove Zn from MT and thus make it more susceptible to proteolytic attack by subtilisin, it is

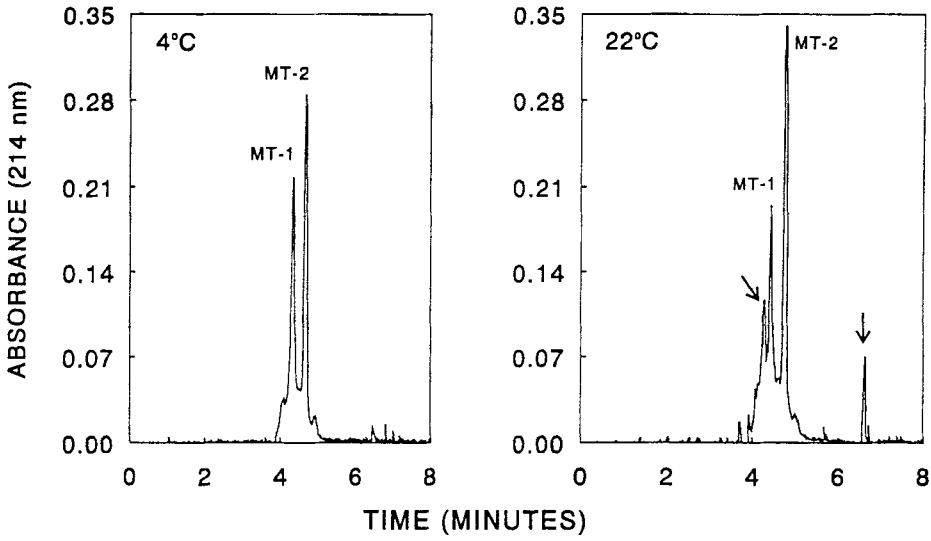

Figure 5. Samples of rabbit liver Cd,Zn-MT (0.5 mg/ml) were maintained either refrigerated at 4°C or at room temperature (22°C). After one month, the stability of the MT samples was assessed using CZE according to the conditions described in the legend for Figure 1. The arrows point to suspected decomposition products that were presumed to have originated from the MT-1 isoform.

apparent that breakdown of MT-1 proceeded over time without prior EDTA treatment. Feldman et al. (20) reported that partial hydrolysis of rat liver Zn-MT did occur at a relatively slow rate *in vitro* with some neutral proteases. Perhaps, EDTA pretreatment accelerates proteolysis by altering the structure of MT. The metal-free form of MT (thionein) is known to be degraded by lysosomal extracts very rapidly compared to the metal-containing form (20). EDTA treatment was also found to be essential to produce a specific fragment of MT containing the α metal-binding domain (residues 30-61) which demonstrates a more positively charged character upon native gel electrophoresis than intact MT (12).

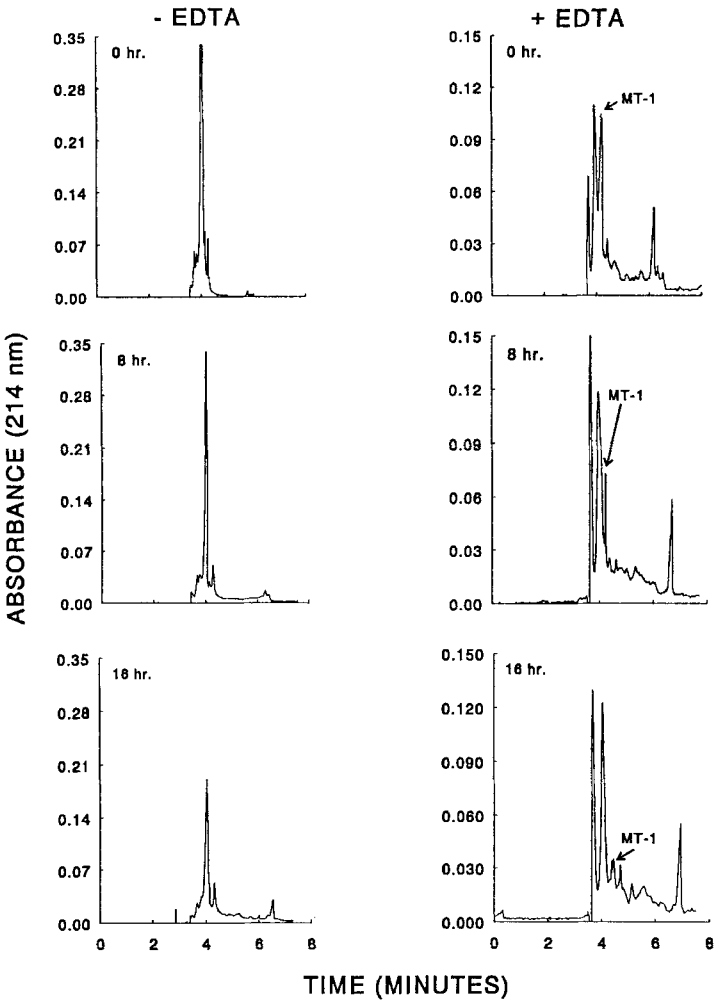

Figure 6. Proteolytic degradation by subtilisin of rabbit liver Cd,Zn-MT-1 preincubated with (+) or without (-) EDTA. Samples were maintained at 4°C during exposure to the protease. The degradation of the MT-1 isoform was monitored at 0, 8 and 16 hr following the addition of subtilisin by CZE according to the conditions described in the legend for Figure 1.

MT-1, subjected to subtilisin digestion in the presence of EDTA, generated peaks more positively charged than intact MT-1 (Figure 6, +EDTA). Similar results were observed for proteolytic degradation of MT-2 and for the combination of both isoforms (data not shown). It is interesting to note that thermal breakdown of rabbit liver Cd,Zn-MT-1 resulted in a peak less negatively charged than intact MT-1 as well as a peak migrating more slowly than MT-1 (see Figure 5). Without appropriate standard materials, it was not possible in this study to characterize these peaks. However, CZE analysis is ideally suited for following the progress of MT degradation since the same sample can be monitored as frequently as is required.

In conclusion, CZE is a new analytical technique useful for the characterization of MT that combines rapid analysis time with superior resolution of MT isoforms. It provides an excellent alternative to other separation techniques especially if sample size is limited or when alternative selectivity is required for resolution of individual MT isoforms. With appropriate sample preparation techniques and standardization, CZE should also prove useful for the quantitative analysis of MT isoforms.

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