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Journal of Chromatography A, 664 (1994) 129–134

JOURNAL OF CHROMATOGRAPHY A

Short Communication

Separation of metallothionein isoforms by micellar electrokinetic capillary chromatography

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(First received June 1st, 1993; revised manuscript received December 28th, 1993)

Abstract

Current techniques for the separation and quantification of metallothionein isoforms have limited value for routine analysis. Isoforms having a similar charge have been separated successfully using reversed-phase HPLC but this technique suffers from a slow sample turnover time. The use of the surfactant sodium dodecyl sulphate for the separation of metallothionein isoforms by micellar electrokinetic capillary chromatography (MECC) is described. The charge-different isoforms MT-1 and MT-2 from rats, rabbits and sheep were separated within 9–12 min. In addition, a varying degree of heterogeneity was observed in purified samples of human MT-1, rat MT-2, rabbit MT-1, rabbit MT-2 and sheep MT-1. The behaviour of chicken MT was different from that of any other species. The separation of sheep liver extracts indicated the potential of MECC as the basis for a quantitative assay for both charge-different and charge-similar metallothionein isoforms.

1. Introduction

Multiple genes for the metal-binding protein metallothionein (MT) have been detected in several mammals [1]. These genes have been cloned, sequenced and a considerable amount is now known about their promoter sites and the regulation of transcription [2–4]. There is, however, virtually no information concerning their translation and the synthesis of the corresponding protein isoforms because of limitations in the methodology for their quantification. Questions concerning the function of individual isoforms cannot therefore be easily addressed.

MT is often measured by isoform insensitive methods such as the Ag [5] or Cd [6] saturation assays although immunological assays specific to the major charge-separable isoforms, MT-1 and MT-2 have been reported [7,8]. The method of choice to study the multiple isoforms of MT-1 and MT-2 is reversed-phase HPLC (RP-HPLC), since the isoforms which do not differ greatly in charge show differences in hydrophobicity [9]. There are however significant disadvantages of RP-HPLC for routine analysis which include the lengthy separation time and the requirement for relatively large sample volumes. A rapid method for the separation and quantification of the major MT isoforms using capillary zone electrophoresis (CZE) has recently been developed

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0021-9673/94/$07.00
SSDI 0021-9673(94)00034-7

[10,11]. Further recent developments indicate that by changing the pH of the electrolyte, it is possible to separate several components from purified MT samples using CZE [12].

Micellar electrokinetic capillary chromatography (MECC) was developed by Terabe and co-workers [13,14] and is a technique using capillary electrophoresis instrumentation with the addition of a micelle-forming ionic surfactant to the electrolyte [15]. The technique has been successfully used to separate proteins [16] so we therefore decided to investigate the separation of MT isoforms and to investigate the potential of MECC for the study of these proteins in tissue extracts.

2. Experimental

2.1. Purified MT samples

Purified metallothionein samples were obtained from several different sources. Rabbit MT (MT-1 and MT-2) and horse MT samples were purchased from Sigma (St. Louis, MO, USA) and rat CdMT-2 and human MT-1 were gifts from Dr. Chiharu Tohyama (National Institute of Environmental Studies, Tsukuba, Japan). Sheep and rat ZnMT isoforms were separated and purified from the livers of animals injected with Zn, using gel filtration and ion-exchange chromatography [17]. Chicken MT was prepared in the same way from birds injected with Cd but was then re-purified by RP-HPLC [18].

2.2. Preparation of liver samples

Chicken livers were obtained from birds which had been injected with Cd. Sheep liver was obtained from a Zn-injected grey-faced ewe [10] and all liver samples were frozen until required. Unless otherwise stated, 100-mg samples of liver were weighed into eppendorf tubes followed by the addition of 200 μl of deionised water. After disruption of the tissue by sonication, 200 μl of acetonitrile were added slowly, while vortexing, to a 200-μl aliquot of the homogenate. The samples were immediately centrifuged at 13 000 rpm (10 000 g) for 5 min and 200 μl of the supernatant were dried using a centrifugal vacuum evaporation system (Speedvac, Savant, NY, USA). The residue was re-dissolved in 20–50 μl of electrolyte buffer prior to analysis.

2.3. Electrophoresis conditions

Samples were analysed using Beckman P/ACE 2100 or 2050 capillary electrophoresis systems fitted with cartridges containing a polyimide-coated fused-silica capillary (Beckman, Fullerton, CA, USA: 50 cm × 75 μm, untreated). Unless otherwise stated, the electrolyte used was 100 mM borate buffer pH 8.4, made by adjusting the pH of a 25 mM solution of sodium tetraborate with 100 mM boric acid, and containing 75 mM sodium dodecyl sulphate (SDS). Samples (0.5–1.0 mg MT isoform ml^{-1} water) were loaded by pressure injection for 1 s and separations were performed at 10 kV and 25°C. Separated components were routinely detected at 200 nm but also at 214, 254 and/or 280 nm using a UV monitor.

In order to evaluate the most suitable concentration of SDS for the electrophoresis of MT samples and liver extracts, separations were performed at a range of different surfactant concentrations and were monitored as described above.

3. Results

3.1. Purified MT samples

Electropherograms for some purified MT samples are shown in Figs. 1–3. Rat MT-2 (Fig. 1), horse MT and rabbit MT-1 (Fig. 2) were both found to contain two components which were well resolved. The purified MT-1 components from sheep and human (Fig. 2) showed evidence of more than two isoforms whereas only one was found in electropherograms of rat MT-1 (Fig. 1), rabbit MT-2 and sheep MT-2 (Fig. 2). The results for chicken MT (Fig. 3) were unlike those obtained with MT samples from other species in

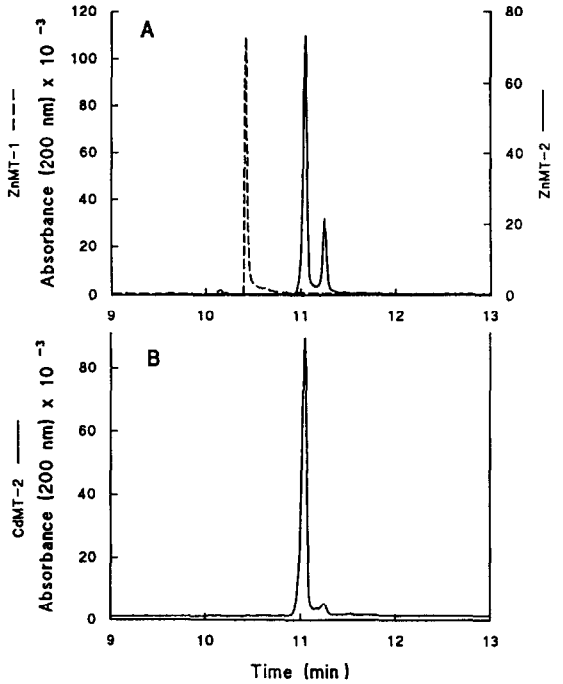

Fig. 1. Separation of purified rat liver ZnMT-1, ZnMT-2 (A) and CdMT-2 (B) (all 1 s injection, 1 mg ml^{-1}) at 10 kV and 25°C using a 57 cm × 75 μm I.D. untreated capillary and a 100 m*M* borate buffer pH 8.4 containing 75 m*M* SDS.

that the resolution of the protein peak was very poor. Variations in migration times of MT-1 and MT-2 between the profiles of purified proteins in Figs. 1–3 may in part be related to species differences in protein structure.

3.2. Liver samples

Preliminary studies to investigate the influence of electrolyte SDS concentration on the separation and resolution of low-M_r components in liver extracts demonstrated that the optimum concentration was between 50 and 75 m*M* SDS (Fig. 4). Migration times increased at higher SDS concentrations which were thought to encourage micelle partitioning and enhance the association of SDS with larger molecules. No improvement in separation or resolution was found by increasing the SDS concentration to 100 m*M* (results not shown) although migration times were further increased. Thus a concentration of 75 m*M* SDS was used routinely in electrolyte buffers.

Standard addition studies with sheep liver extract using purified sheep MT (not shown) had the effect of increasing the absorbance peaks corresponding to components in the purified MT alone. By monitoring at different wavelengths, it was possible to obtain some spectral information concerning all separated components in the native sheep extract (Fig. 5). These profiles demonstrated that the components labelled a, b and c in Fig. 5 showed little absorbance at 280 nm, indicating a low aromatic amino acid content which is characteristic of MT.

4. Discussion

The critical micelle concentration for SDS is reported to be 8.2 m*M* [19] although a much higher concentration is often used for the optimal separation of compounds by MECC [15]. We found that a concentration of 75 m*M* gave the optimum separation for chicken liver extract (Fig. 4) and that these conditions were also suitable for sheep liver extract (Fig. 5). As the SDS concentration was increased from 25 to 75 m*M*, the migration time also increased (Fig. 4) indicating a greater partitioning of the extract components into the micelles.

The electroosmotic front (EOFront) marks the position of zero net charge, which in the present work was observed at 8.5–9.0 min. MT-1 proteins were found to migrate at 1.1–1.2 × the EOFront with MT-2 proteins following at 1.2–1.3 × the EOFront. In comparison to RP-HPLC, the migration times and sample turnover times for MT isoforms were very favourable. The charge-separable isoforms referred to as MT-1 and MT-2 due to their sequential separation on conventional ion-exchange chromatography, often showed evidence of heterogeneity which could indicate the presence of additional isoforms. However, there are a number of other possible explanations including sample contamination, degradation/aggregation or the formation of different metalloforms. Analysis of the purified proteins by laser desorption ionisation

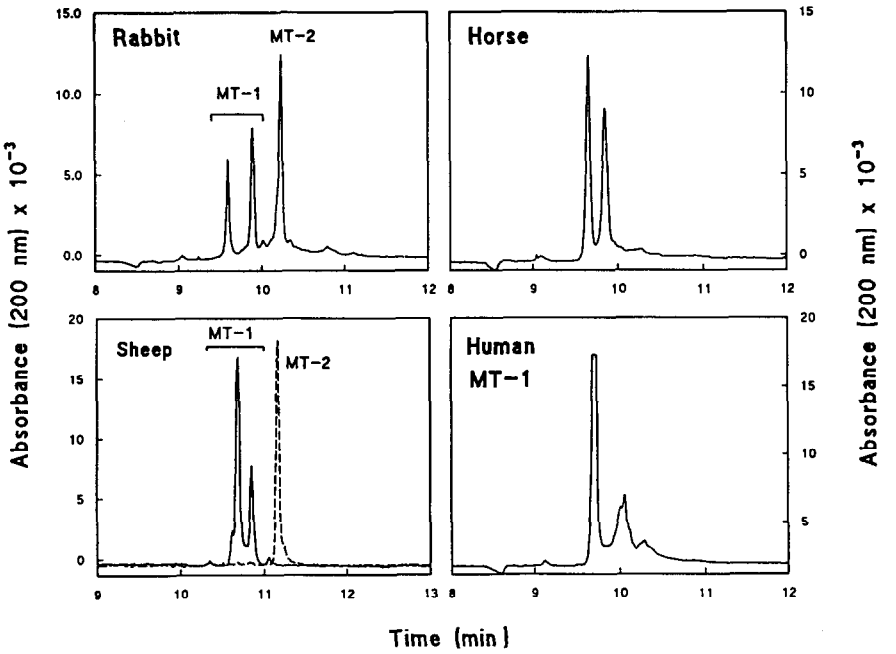

Fig. 2. Separation of rabbit and sheep liver MT, human liver MT-1 and horse kidney MT (all 1 s injection, 1 mg ml^{-1}) using the conditions described in Fig. 1.

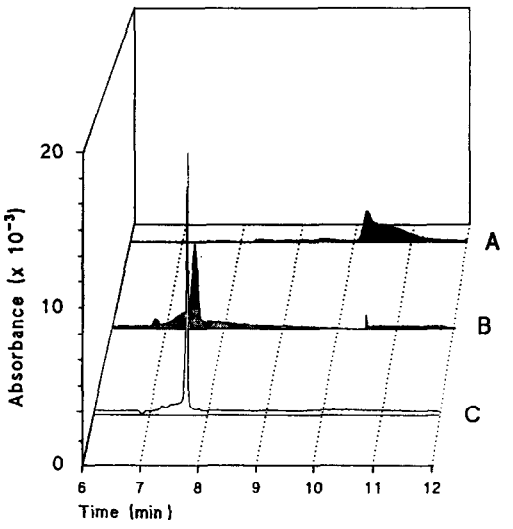

Fig. 3. Separation of purified chicken liver CdMT (A) at 10 kV and 25°C. SDS was ommitted from the electrolyte but not the sample buffer (B) and from both the electrolyte and the sample buffer (C). All samples contained 0.5 mg MT ml^{-1} (1 s injection).

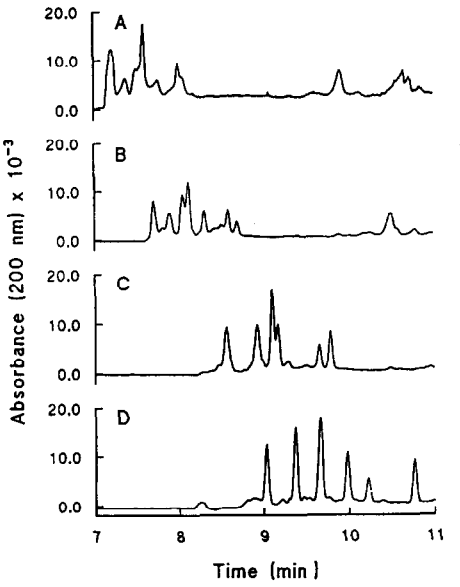

Fig. 4. Effect of increasing the electrolyte SDS concentration on the separation of low-M_r components in a chicken liver extract. The electrolytes contained 100 mM sodium borate pH 8.4 and (A) 0, (B) 25, (C) 50 and (D) 75 mM SDS.

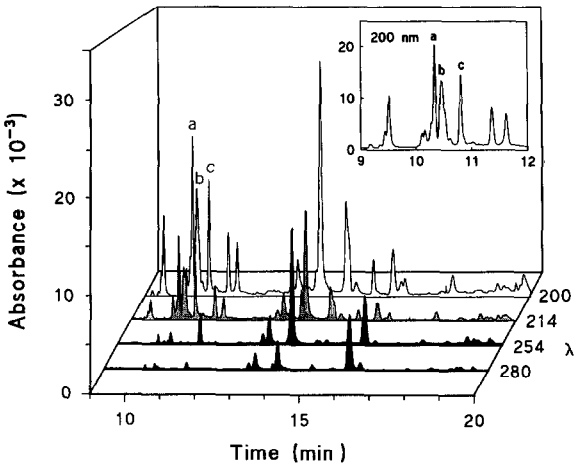

Fig. 5. Separation of sheep liver extract at four different wavelengths using the conditions described in Fig. 1 and detail of the MT peaks at 200 nm are shown inset. Peaks a and b contain MT-1 species whereas peak c is MT-2.

mass spectrometry did not indicate contamination or degradation/aggregation with the possible exception of horse MT. Sheep have at least three functioning MT-1 genes [20] and amino acid analyses of the hepatic protein gave a composition characteristic of MT. This supports UV spectral data showing low absorbance at 280 nm and indicating the absence of aromatic amino acids in all the MECC separated components. Separation of sheep MT-1 by CZE at pH 2.5, 7.0 and 11.0 in each case gave a single, well resolved absorbance peak [12] and this indicates homology of acidic and basic amino acid residues between the two main components separated by MECC. SDS is reported to encourage the formation of inter- and/or intramolecular linkages of MT through cysteine groups, resulting in broad obscure bands with SDS–polyacrylamide gel electrophoresis [21]. However, in all but one MT sample, the components separated using SDS-MECC were of high resolution and were highly reproducible both as regards component migration time and peak area. The single exception was chicken MT which appeared to be adversely affected by the SDS in the sample buffer and/or in the electrolyte. Chicken MT is unusual in that it contains two additional amino acids and the terminal carboxyl residue is a histidine [22]. Thus its uncharacteristic behaviour with MECC conditions may be related to these modifications.

Finally, we investigated the possibility that the various components observed when separating purified MT isoforms could be metalloforms of the same protein but found, at least as regards rat (Fig. 1) or rabbit (not shown) ZnMT-2 and CdMT-2, that there was no difference in migration time relating to the species of metal bound.

The separation of purified rat ZnMT-2 showed two components, one major and one relatively minor which concurs with the previous evidence for two Zn-containing components in rat MT-2 samples separated by ion-exchange HPLC [23]. Two components have also been isolated from mouse liver MT-2 [24], although there is as yet no evidence for three functioning MT genes in these rodents. The occurrence of 2 or more isoforms of MT-2 would be unusual since most of the heterogeneity in many species occurs in the MT-1 form. CdMT-2 was prepared from Sprague–Dawley rats in a Japanese laboratory, separated using the same conditions as for the rat ZnMT-2 (Hooded Lister rats) and the electropherogram also showed evidence of a component with a similar migration time (Fig. 1B), albeit at lower concentration. The appearance of a similar component in MT-2 specifically from Zn-treated mice has been noted previously [23].

The results of rabbit MT separations show two major MT-1 components and one prominent MT-2 protein. These results are in agreement with CZE electropherograms of rabbit MT-1 and MT-2 proteins at low pH which show a total of three prominent components [12]. There is evidence for the existence of six MT isoforms in rabbit tissues, some of which are minor as regards their cellular concentration but which nevertheless show relatively high levels of induction by metals [25]. The separation of horse MT was unusual since the difference in migration time between the two partially separated components was uncharacteristic of that normally found for MT-1 and MT-2. The CZE separation of horse MT at pH 11 showed three distinct components indicating the presence of one or more proteins with a charge uncharacteristic for MT at this pH [12]. The heterogeneity of human

MT-1 is well known [26] and we have partially resolved three of the proteins so far identified.

Using the MECC conditions described here, we have separated smaller related peptides such as oxidised and reduced glutathione, which migrate at approximately 1.5 and 1.6 × the EO-Front, respectively (data not shown). Although we have not yet identified many of the components separated by MECC of liver extracts, there is clearly great potential for the simultaneous quantification of low-M_r cell components including metallothionein and glutathione. The acetonitrile extraction procedure which removes most proteins and complex carbohydrates is simple, rapid and compatible for direct analysis by MECC. Investigations are continuing on improvements to the preparation procedure and assay conditions which will permit the complete separation of MT isoforms from contaminating components in tissues from a wide variety of different animal species. We conclude that MECC is a useful method for the separation of MT isoforms and that this technique shows considerable potential for their rapid quantification.

5. Acknowledgements

The authors wish to thank Dr. Ian Bremner for his critical review of the manuscript. This work was funded by the Scottish Office Agriculture and Fisheries Department, UK and the US Department of Agriculture.

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