Separation and Identification of Metallothionein Isoforms by CZE-PDA

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Introduction

Metallothioneins (MTs) are a polymorphic group of non-enzymatic low molecular mass proteins (6–7 kDa), characterised by their resistance to thermocoagulation and acid precipitation, by a high cysteine content (~30 %), and the lack of aromatic amino acids. MT are proteins induced by various stimuli such as metals, stress and some drugs. MT has the ability to bind with high affinity and stoichiometry to d^{10} metals such as zinc, cadmium, copper and mercury [1–3]. The MTs seem to play an important role in number of metabolic mechanisms including the homeostasis of some essential metals (Zn, Cu) and detoxification of harmful (Cd, Pb) and excessive essential metals [4]. MT isoforms are the product of genetic polymorphism characteristic of MT genes in animals and humans. Considering their electrophoretic properties, two major isoforms of MT have been identified in mammals, MT-1 and MT-2, named after their order of elution in anion-exchange chromatography. They have a single charge difference at neutral pH due to certain amino acid substitutions [5]. Furthermore, many animal species generate various subisoforms of MT-1 and MT-2, therefore displaying significant microheterogeneity.

Investigation of the functional significance of the individual MT isoforms and subisoforms requires analytical techniques that offer a high degree of resolution. Recently, CZE using uncoated capillaries has shown promising results [6–11]. In a previous paper we introduced the use of CZE with the mixed tris-borate buffer at neutral or nearly neutral pH [10]. The study was performed under the original conditions where the metals are still bound to molecules unlike at acidic pH values where the metals are dissociated. In this paper we study the use of photodiode array detection (DAD) to obtain more information about the separated putative isoforms. The use of DAD gives the possibility obtaining a UV spectrum of the separated peaks thus enabling further characterisation of the isoforms and better understanding of their behaviour.

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Results and Discussion

Separation of Metallothionein Samples

Horse kidney exhibits 5 major peaks when separated using a mixed tris-borate buffer at pH 6.90 (Figure 1A) [12]. The horse kidney MT peaks show two types of UV scans. Peaks labelled A–C in Figure 1A give UV spectra corresponding to compounds totally lacking or nearly totally lacking cation-thiol bondage. Characteristic wavelengths for cation-thiol bondage found in metallothioneins are 225 nm for Zn-thiol and 250 nm for Cd-thiol [2, 13]. This may indicate that these three peaks of horse kidney MT labelled A–C in Figure 1A are in apothionein form or contain only very little metal. This observation is very surprising since at this pH the metals should be bound to the protein and to find apothioneins in tissues at neutral pH is unexpected. Horse kidney MT peaks labelled D and E in Figure 1A give typical spectra for metallothioneins containing zinc and cadmium.

Rabbit liver MT (Figure 1B) shows 3 major peaks and several smaller ones. Rabbit liver MT-1 (Figure 1C) gives 2 major peaks and 5 smaller ones. Rabbit liver MT-2 (Figure 1D) exhibits one major peak and 6 smaller ones. In the rabbit liver MT sample, which is theoretically a mixture of both rabbit liver MT-1 and MT-2, it is likely that all the peaks of MT-1 and MT-2 should be observed.

The rabbit liver MT main peak labelled 5 as well as peaks labelled 6 and 11 in Figure 1B were found to give UV spectra corresponding to metallothioneins containing zinc and cadmium bound to thiol groups. The rabbit

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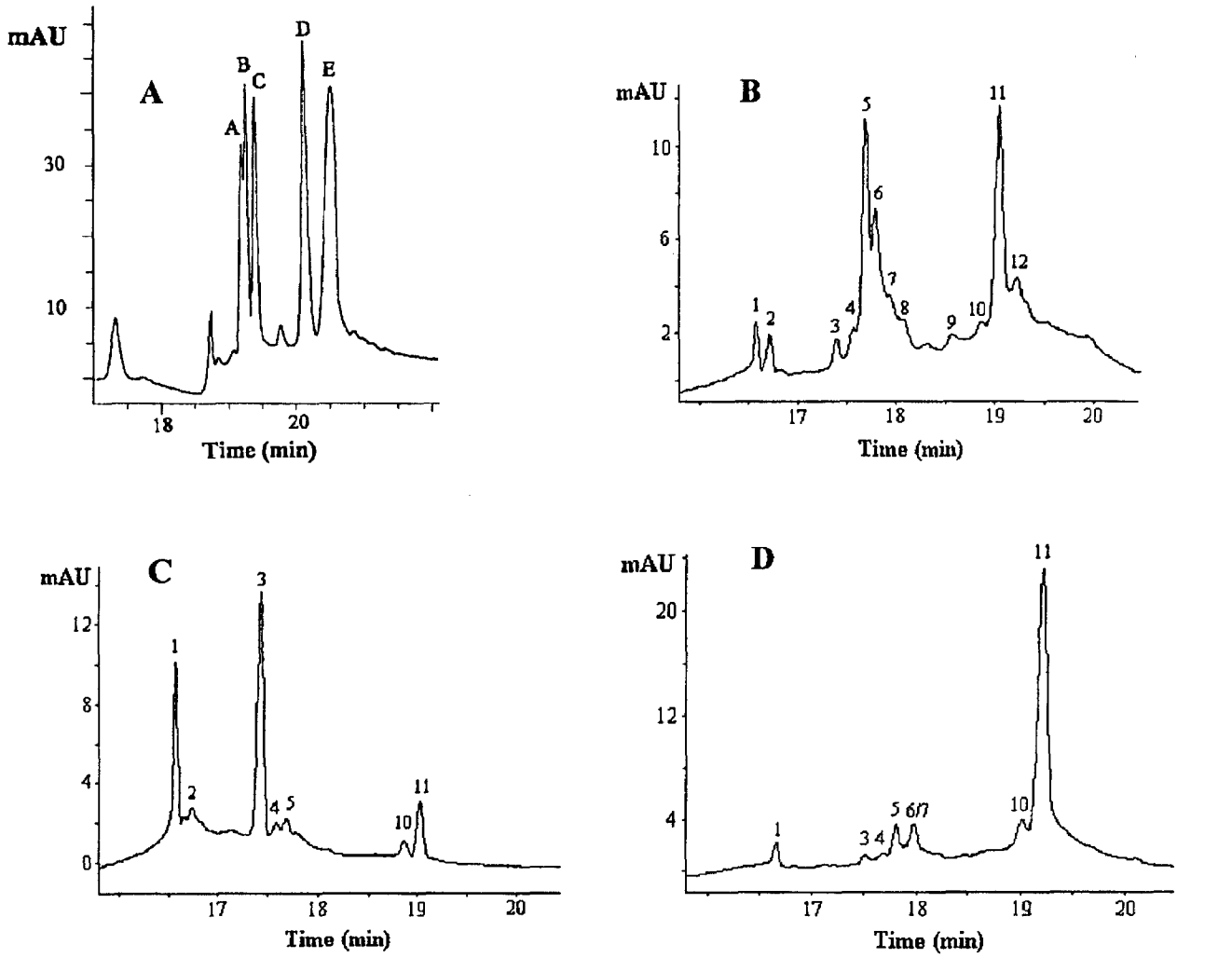

Figure 1

Electropherograms of horse kidney MT (A), rabbit liver MT (B), rabbit liver MT-1 (C) and rabbit liver MT-2 (D). Conditions: Tris-borate buffer 110 mM–110 mM, pH 6.90 in A and 6.40 in B–D, 20 °C and 10 kV in neutral polarity. Reprinted from Virtanen et al., J. Liq. Chromatogr. 21 (1998) p. 3087 by courtesy of Marcel Dekker Inc.

liver MT-1 main peaks labelled 1 and 3 in Figure 1C also gave similar UV spectra as well as the rabbit liver MT-2 main peak labelled 11 in Figure 1D. No peaks observed in rabbit liver samples gave UV spectra typical for apothioneins.

Electrophoretic Mobilities of Metallothionein Isoforms

Effects of buffer pH, buffer concentration and buffer composition on electrophoretic mobilities of horse kidney MT peaks labelled B, D and E in Figure 1A are shown in Tables I–III. Peak B was chosen to model the behaviour of peaks A–C since they all gave similar UV spectra. The electrophoretic mobility of all peaks increased slowly with increasing buffer pH (Table I), but still a difference in behaviour of peak B was observed compared to others. The electrophoretic mobility was lower for peak B than for peaks D and E. As the peak B was found to be either in apothionein form or containing very little metals its electrophoretic behaviour is likely to be affected differently than metal-saturated metallothionein isoforms as pH changes. Rabbit liver MT peaks labelled 5 and 11 in Figure 1B were chosen for electrophoretic studies. The behaviour of these peaks is very similar to those of horse kidney peaks D and E (Table I) when buffer pH is varied. The one charged isoform, MT-1, labelled as peak 5 in Figure 1B–D has very similar electrophoretic mobility as horse kidney peaks D and E, and the 2-charged isoform, MT-2, labelled as peak 11 in Figure 1B–D had logically higher mobility.

An interesting phenomenon was observed when the concentration of the mixed tris-borate buffer was varied (Table II). Nearly constant electrophoretic mobility of peak B was noticed as the concentration of the mixed tris-borate buffer increased from 50 mM–50 mM to 150 mM–150 mM. The well known [14–15] tendency of decreasing electrophoretic mobility with increasing buffer concentration was observed both for horse kidney MT peaks labelled D and E in Figure 1A and rabbit liver MT peaks labelled 5 and 11 in Figure 1B. RL MT-1

Table I. Effect of the mixed tris-borate buffer pH on the electrophoretic mobities (μ_{ep}) of horse kidney (HK) peaks B,D and E and rabbit liver (RL) peaks 5 and 11.

Buffer pH	HK B	HK D	HK E	RL 5	RL 11
5.52	0.138	0.251	0.282	0.291	0.417
6.47	0.198	0.288	0.314	0.316	0.446
6.90	0.212	0.299	0.319	0.323	0.455
7.20	0.256	0.318	0.340	0.343	0.479
7.60	0.183	0.305	0.319	0.353	0.512
8.01	0.197	0.337	0.350	0.362	0.521
8.50	0.223	0.403	0.403	0.420	0.618

$\mu_{ep} = (\times 10^8\ m^2/V \cdot s)$

Table II. Effect of the mixed tris-borate buffer concentration on the electrophoretic mobities (μ_{ep}) of horse kidney (HK) peaks B,D and E and rabbit liver (RL) peaks 5 and 11.

Buffer conc.(mM)	HK B	HK D	HK E	RL 5	RL 11
50–50	0.237	0.377	0.394	0.404	0.569
70–70	0.227	0.342	0.361	0.370	0.521
90–90	0.225	0.321	0.342	0.347	0.488
110–110	0.216	0.297	0.320	0.326	0.455
130–130	0.219	0.291	0.314	0.315	0.441
150–150	0.213	0.277	0.302	0.305	0.424

$\mu_{ep} = (\times 10^8\ m^2/V \cdot s)$

Table III. Effect of the mixed tris-borate buffer composition on the electrophoretic mobities (μ_{ep}) of horse kidney (HK) peaks B,D and E and rabbit liver (RL) peaks 5 and 11.

Buffer comp.(mM)	HK B	HK D	HK E	RL 5	RL 11
110–30	0.211	0.281	0.308	0.312	0.442
110–50	0.213	0.287	0.313	0.317	0.449
110–70	0.216	0.293	0.318	0.322	0.450
110–90	0.219	0.299	0.323	0.327	0.459
110–110	0.216	0.297	0.320	0.326	0.455
90–110	0.236	0.326	0.346	0.353	0.494
70–110	0.270	0.359	0.377	0.380	0.532
50–110	0.233	0.386	0.398	0.412	0.577
30–110	0.206	0.427	0.454	0.457	0.635

had nearly similar mobility as horse kidney peaks D and E (Table II) and RL MT-2 had similarly higher mobility as in the case of the effect of the buffer pH.

The buffer composition had little effect on the electrophoretic mobility of horse kidney MT peak B (Table III). The tendency for horse kidney MT peaks D and E and rabbit liver MT peaks is similar to peak B up to buffer concentration of 110 mM tris–110 mM borate. When borate concentration remained at 110 mM and tris concentration was decreased a slight increase in electrophoretic mobilities was observed which is different to the behaviour of peak B. This effect is interesting and more studies should be done to characterise this behaviour.

Conclusions

Four mammalian metallothioneins (MTs), rabbit liver MT (RL MT), rabbit liver MT-1 (RL MT-1) and rabbit liver MT-2 (RL MT-2) and horse kidney MT (HK MT), have been subjected to capillary zone electrophoresis (CZE) with on-line photodiode array detection (DAD) under neutral or nearly neutral conditions. Differences in electrophoretic behaviour were observed. As expected, the two charged main peak of RL MT-2 showed the highest electrophoretic mobility. The main peak of RL MT-1 and two of rabbit liver MT had lower electrophoretic mobilities corresponding to single charge. The UV spectra of the main peaks of rabbit liver MT and those of rabbit liver MT-1 and MT-2 were typical for metallothioneins containing shoulders for Zn-thiol and Cd-thiol bondage at 225 nm and 250 nm, respectively.

The two later migrating horse kidney MT peaks had nearly similar electrophoretic behaviour to the single charged rabbit liver MT peaks. Interestingly, horse kidney MT also exhibited peaks with lower electrophoretic mobilities and different behaviour thus differing from the other peaks observed. These peaks showed no absorbance or nearly no absorbance at 225 and 250 nm that correspond to , Zn-thiol and Cd-thiol, respectively. We can therefore assume, that the horse kidney MT sample contains components that are in apothionein form or non-saturated with metals. This information is interesting since at the pH used metals should not be dissociated. The identification of these peaks as true metallothionein isoforms awaits further characterisation. The use of photodiode array detection appears to be a good tool for a quick identification of apothioneins, partially metal-saturated metallothioneins and metallothioneins.

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