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Improved separation of metallothionein isoforms by the presence of cyclodextrin in capillary zone electrophoresis

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Abstract

Capillary zone electrophoresis (CZE) with cyclodextrin (CD) in the polyacrylamide-coated capillary was used to study metallothionein (MT) forms in the horse kidney preparation produced commercially by Sigma. It is known that CDs form complexes with hydrophobic amino acids. The results show that the presence of CD improves the separability of the various MT forms, including the MT-IA and the MT-IB forms, metallothionein aggregates, as well as the so far unidentified a and b forms. This was true both below and above the isoelectric points (p*I*s), although the migration times were somewhat longer at increasing CD concentrations for runs at constant voltage than with constant current.

Keywords: Metallothionein isoforms; Cyclodextrin; Acrylamide-coated capillaries; Capillary zone electrophoresis.

1. Introduction

Metallothionein (MT) has, since its discovery by Margoshes and Vallee [1], been thoroughly discussed due to its unike chemical and physical properties and because of its fundamental impact on heavy metal detoxification. In later years, it has also been understood that it plays an important role in the inactivation of reactive oxygen species (ROS) in the body.

This protein has a peculiar amino acid composition having a very high cysteine content and it lacks aromatic amino acids and histidine. In most animal species, MTs are present in two main isoforms (MT-I and MT-II) with isoelectric points of about 3.9 and 4.6, respectively. Horse kidney MT, however, being studied in the present work, is present in two main forms, MT-IA and MT-IB, with corresponding isoelectric points (p*I*s) [2]. Heavy metals are strongly bound to the protein in the pH interval of 5–11. Their molecular weights are about 6100, when bound to metal 6800 Da. The cystein chains can bind 7.2 g atoms of divalent heavy metal pr mole of protein which amounts to 6–11% of the total molecular weight.

The MT forms generally consist of 15 different amino acids arranged in a total composition of 50–70 amino acid units. Both isoforms have 30–35% cysteins, constituting about 20 units, which are coordinated in seven groups, including one Cys–X–Cys, three Cys–X–X–Cys and three Cys–Cys groups. The metal binding thiols are oriented in two domains within the protein, each binding three or four divalent metal ions, respectively. Metal binding occurs in trimercaptide complexes preferably with divalent heavy metals like Cd, Zn, but also Ag, Au, Cu, Hg and Pt. These particular metals are not only strongly bound, but are also potent inducers of the protein in exposed organisms. Glucocorticoids (e.g. dexamethazone) as well as certain illnesses and stress situations may also induce the protein, which may be related to ROS inactivation [3].

Capillary zone electrophoresis (CZE) in the polyacrylamide-coated capillary has previously been used in our laboratory [2,4,5] to study MT isoforms as well as non-MT components in the horse kidney and rabbit MT preparations produced commercially by Sigma. This technique was found to be well suited for studies of such forms both at different

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pHs and in various buffer systems. The non-MT forms, named by us as the a and b forms, are heat resistant, they do not seem to bind metal, but comigrate with MT in the various systems generally used for MT isolations.

The molecular components in a sample can be separated by carrier free electrophoresis only if the difference in charge (zeta potential) is sufficiently large. A common way to increase such differences is to supplement the electrophoresis buffer with agents that complex with the sample components thereby changing their electric charge. It is regarded, however, that it is impossible to predict whether such complexation may increase the resolution [6]. Complexing agents used for this purpose are many, but in the present work on MT separation we have focused on the various types of cyclodextrins (CDs) [6,7]. Cyclodextrins form uncharged complexes with hydrophobic amino acids, thereby increasing the relative solubility of hydrophobic organic compounds [8–10].

The most widely used α-, β- and γ-CDs are truncated cone-shaped molecules with a hollow, tapered cavity of 7.9 Å depth. The top and bottom diameters of the cavities are 4.7 and 5.3 Å for α-CD, 6.0 and 6.5 for β-CD and 7.5 and 8.3 Å for γ-CD, respectively [11–13]. The α-, β- and γ-CDs consist of six, seven and eight D-glucopyranose residues, respectively, which are linked by α-1,4 glycosidic bonds into a so-called macrocycle [13]. The most probable mode of binding involves the insertion of the hydrophobic part of the guest molecule into the cavity, while the polar group of the guest is exposed to the bulk solvent surrounding the wider opening of the cavity [12]. The inclusion of the guest molecule by CD in aqueous solution results in a substantial rearrangement of water molecules originally solvated to both the CD and the guest molecule. This process also releases water molecules from the CD cavity into the bulk water. The binding are primarily believed to be caused by Van der Waals forces and hydrophobic interactions, although hydrogen bonds and steric effects also are involved [12–15].

The subject of the present work was to study the effect of CDs as buffer additives on the resolution ability of the MT forms in CZE. In the present work we have also used an improved acrylamide coating of the capillaries used.

2. Experimental

2.1. Chemicals

Horse kidney MT (M4766), containing 4.8% Cd and 0.8% Zn (lot 73H9544) and claimed to be essentially salt free, was obtained from Sigma, St. Louis, MO, USA. The commercial MT preparation was dissolved in 10 mM Trizma pre-set crystal buffer at pH 9.1 to a final concentration of 180 μg/ml and kept as stock solution at −75 °C under nitrogen. The CDs used in the present experiments are listed in Table 1.

Trizma pre-set crystals (pH 9.1) were also bought from Sigma. Bind-Silane (γ-methacryloxy-propyl-trimethoxysilane) came from Amersham Pharmacia Biotech, Uppsala, Sweden. Acrylamide (99.9% pure), ammonium persulfate (>99% purity) and TEMED (*N*,*N*,*N*′,*N*′-tetramethylenediamine) were bought from Bio-Rad Labs, Life Science Group, Richmond, CA, USA. Fluorinated liquid (FC-77) was obtained from 3M, Chemical Group, Haven, Belgium. Orthophosphoric acid (H_3PO_4, 99% pure cryst.), acetone, HCl and NaOH came from E. Merck, Darmstadt, Germany. Cernite No. 1 was obtained from T + F GmbH, Dreieich, Germany. All chemicals were of the highest available purity. Buffers and solutions were degassed before use at 3–6 bar and filtrated through a 0.45 μm filter (Millex-HA, Millipore, Bedford, MA, USA). The solutions were prepared weekly.

2.2. Capillary zone electrophoresis (CZE) of MT samples

The BioFocus 3000 Capillary Electrophoresis System with Spectra Software version 3.00, Integration Software version 3.01 and BioFocus Capillary Cartridge from Bio-Rad were used for MT analysis. Silica capillaries, HT-fused from MicroQuartz, Munich, Germany, 24 cm × 25 μm inner diameter (19.3 cm to the detection window) were coated with polyacrylamide in our laboratory to suppress electroendosmosis and adsorption of the analytes onto the wall of the capillary. The procedure started out by using a long capillary

Table 1
Cyclodextrins used in the present experiments

Source	Cyclodextrin (CD)	Identity no.	Lot	M.S.M.*	Charge
Sigma	2-Hydroxypropyl-β-CD	C-0926	032K1553	4.9	Neutral
	Heptakis(2,6-di-O-methyl)-β-CD	H-0513	18H0272	14–15	Neutral
	Heptakis(2,3,6-tri-O-methyl)-β-CD	H-4645	94H0659	21	Neutral
	6-Monodeoxy-6-monoamino-β-CD	M-2314	043K0802	1	Positive
	γ-CD polymer	C-2860	013K0575	–	Neutral
Aldrich	α-CD hydrate	85,609-6	S10381–502	–	Neutral
	2-Hydroxypropyl-α-CD	39,069-0	32821–023	3.6	Neutral
	α-CD hydrate, sulfated, Na-salt	49,454-2	19312BA	12	Negative
	β-CD, sulfated, Na-salt	38,915-3	05706HI	7-11	Negative
	Hydroxypropyl-β-CD	38,914-5	14303PU	5	Neutral
	Hydroxypropyl-β-CD	33,259-3	17202TS-153	4	Neutral
	Hydroxypropyl-γ-CD	39,070-4	S01318-052	5	Neutral

* M.S.M. = molar substitution pr mole CD.

Table 2
Stepwise procedure for capillary coating

Step no.	Substance	Washing procedure[a]
1	Aq. dest.	20
2	0.5 M NaOH	5
3	Aq. dest.	10
4	0.5 M HCl	5
5	Aq. dest.	10
6	Acetone	5
7	Nitrogen gas	5
8	Bind-Silane 50%[b,d]	10
9	Acetone	5
10	Aq. dest.	10
11	Nitrogen gas	10
12	Acrylamide 4%[c,d]	10
13	Aq. dest.	20
14	Nitrogen gas	20

[a] Washings and introduction of substances are in minutes and performed at a pressure of 11 bar.
[b] Bind-Silane contains 50% acetone.
[c] Four percent acrylamide containing 1 μl TEMED and 1 mg ammonium persulfate/ml.
[d] Capillary sealed in both ends with Cernite No. 1 for 20 h.

of 1.5 m with i.d. of 25 μm and o.d. of 365 μm, enough for six individual capillaries of 24 cm each. To allow spectrophotometry windows were burnt out in the outer supporting polyimide coating of the capillary prior to coating by the use of a Window Maker from Micro Solve, Long Branch, USA. See Table 2 for the stepwise coating procedure. The use of Bind-Silane and acetone in a 50–50% mixture and 4% acrylamide proved to give an improvement compared to previous coating procedures used in our laboratory [2,5]. The durability of the coated capillaries were increased by keeping them at 4–6 °C.

The CZE experiments were performed in sodium phosphate buffers of 500 and 100 mM, at pH 1.5 and 7.4, respectively. At pH 1.5 the individual experiments were done either at constant voltage (10 kV), giving a maximum current of 65 μA in the capillary, or at constant current (65 μA) giving a maximum of 16 kV in the capillary. Above the p*I* at pH 7.4, the individual experiments were done at constant voltage (15 kV), giving a maximum current of 78 μA, or at constant current (78 μA), giving a maximum of 20 kV in the capillary. All samples were applied hydrodynamically by pressure (15 p.s.i. × s; 1 p.s.i = 6894.76 Pa) and analysed with polarity from positive to the negative electrode below the isoelectric points (p*I*s), the opposite way above p*I*. The negatively charged CDs were analysed with polarity from negative to the positive electrode at low pH. The cartridge and the carousel were thermostated at 20 °C by the Peltier thermoelectric cooling system with fluorinated liquid. Prior to runs on the capillary column, the MT samples were kept in 10 mM Trisma pre-set buffer at pH 9.1. In this way the change to running pH conditions occurred immediately before the actual run. At this high pH the metal will stay securely on the protein, thereby also protecting it from configurational changes and deterioration. The washing procedure for the system between each run was as follows: first, 1 M HCl for 25 s, then aq. dest. for 15 s, and finally, electrophoresis buffer for 60 s. Absorbance was monitored at 200 nm at pH 1.5 and at 200, 254 and 280 nm at pH 7.4.

3. Results and discussion

The results found in the present work show that presence of several CDs in the capillary running buffer generally gives better results with respect to peak separability, peak heights and baseline stability for the various MT forms as well as for the a and b forms of the horse kidney MT preparation. A marked concentration dependent increase in migration times for the various forms was observed, however, which may be regarded as inconvenient. Among our tested CDs very good results at pH 1.5 were obtained for heptakis (2,6-di-*o*-methyl)-β-CD for which an increase in separability occurred already at 0.5% concentration in the capillary buffer. At pH 7.4 the best registrations were obtained for 2-hydroxypropyl-α-CD. Sets of registrations are given in Figs. 1 and 2. It can be seen that the a and b forms are considerably better separated in the presence of CD, and that the migration times of the various forms are shorter with and without CD present for constant current than for constant voltage.

The efficient separation abilities of the CDs can be attributed to decreased diffusion coefficients due to formation of aggregates and reduced adsorption to the capillary wall [6]. The acrylamide coating of the capillaries were not hampered by the presence of CDs in the running buffer, and the long lasting capillaries could conveniently be kept by storing in water overnight. This has also been observed previously [6].

The results obtained for the other CDs tested at pH 1.5 are not shown in detail in the present work. Generally, however, it can be said that these CDs are not as effective as the heptakis (2,6-di-*o*-methyl)-β-CD when speaking of separability and baseline stability. The a forms were not completely baseline separated for the hydroxypropyl-β-CD, for hydroxypropyl-γ-CD and for 6-monodeoxy-6-monoamino-β-CD, all measured at 4% of their respective CDs. For the two latter CDs the b1 form could hardly be seen. In the case of the 6-monodeoxy-6-monoamino-β-CD the MT-B forms were incompletely separated. The poor separation ability at both pH 1.5 and 7.4 for the sulphated, negatively charged β-cyclodextrin (not shown) may be due to steric hindrance of the guest MT, because the molar substitution value for this particular CD is claimed to be as high as 7–11 (Table 1) [8–10].

Registrations at pH 1.5 by constant current of 65 μA gave somewhat better separations than for constant voltage of 10 kV with higher peak heights and shorter migration times. The current flow of 65 μA was the highest value observed when samples were run at 10 kV constant voltage, which means that there is a somewhat higher current flow in the capillary at 65 μA than at 10 kV constant voltage. The CDs tested undergo no charge changes throughout the pH inter-

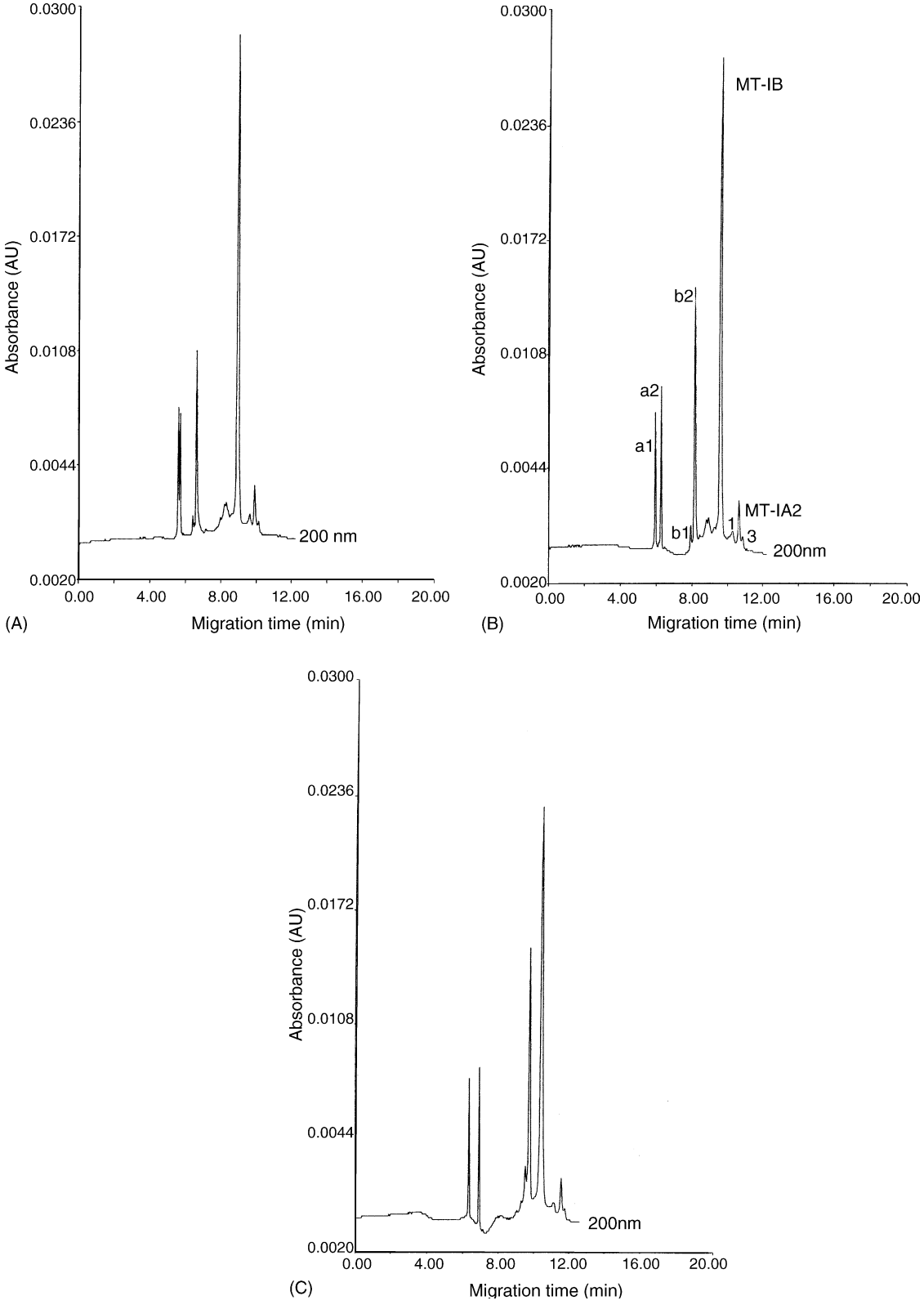

Fig. 1. CZE of the horse kidney MT preparation at 500 mM phosphate buffer, pH 1.5, 10 kV constant voltage. Polarity + to −. Sample size applied to the capillary in each run 8.4 nl of stock solution 180 μg/ml buffer. The identification of the various peaks are given in (B): (A) CD not added; (B) 2% heptakis (2,6-di-*o*-methyl)-β-CD present in the running buffer; (C) 4% heptakis, otherwise as in (B).

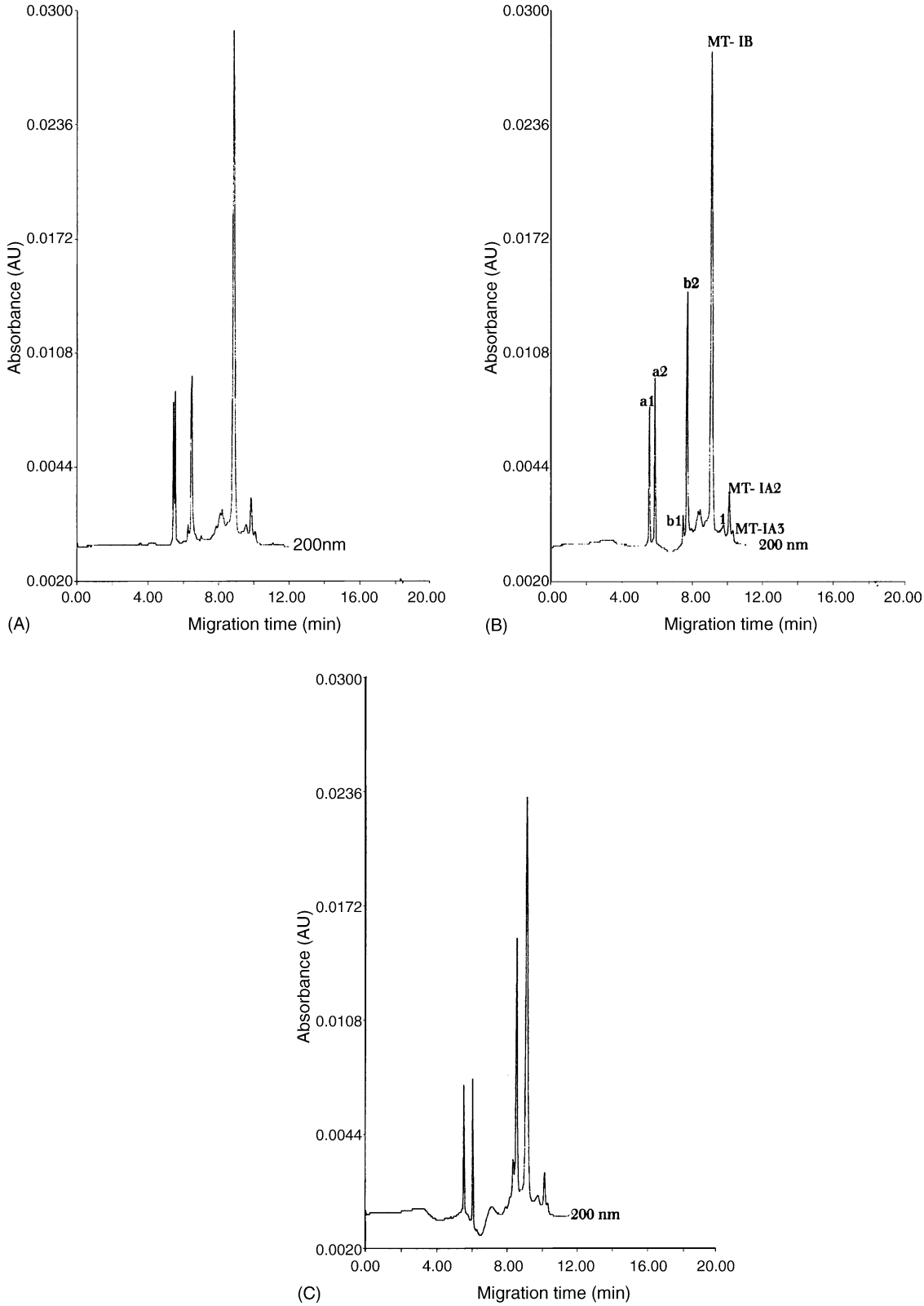

Fig. 2. CZE of the horse kidney MT preparation at 500 mM phosphate buffer, pH 1.5, 65 μA constant current. Polarity + to −. Sample size applied to the capillary in each run 8.4 nl of stock solution 180 μg/ml buffer. The identification of the various peaks are given in Fig. 1(B): (A) CD not added; (B) 2% heptakis (2,6-di-*o*-methyl)-β-CD present in the running buffer; (C) 4% heptakis, otherwise as in (B).

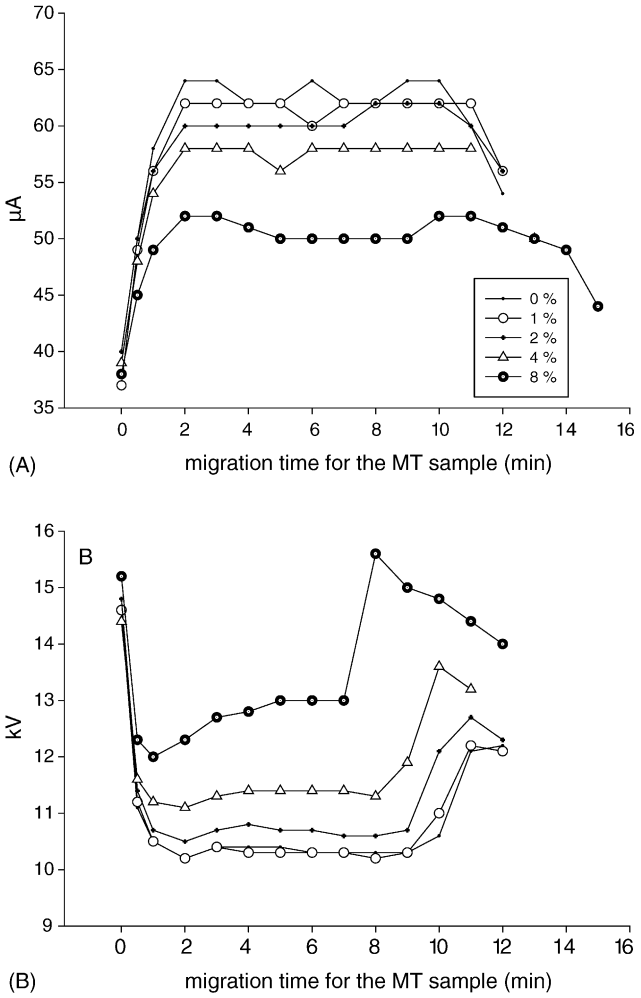

Fig. 3. Current and voltage monitored in the capillary at constant voltage and current, respectively, in CZE of the horse kidney MT preparation at 500 mM phosphate buffer, pH 1.5. Polarity + to −. Sample size applied to the capillary in each run 8.4 nl of stock solution 180 μg/ml buffer. (A) Current monitored with 0–8% 2-hydroxypropyl-α-CD at constant voltage, 10 kV. (B) Voltage monitored with 0–8% 2-hydroxypropyl-α-CD at constant current, 65 μA.

val being studied, and therefore it is understandable that the conductance of the capillary buffer is reduced when CD concentrations are increased. At constant voltage, therefore, the current goes down and the migration times become longer. Fig. 3(A) shows the current flow in the capillary at constant voltage (10 kV) for 2-hydroxypropyl-α-CD at various migration timepoints for the MT sample. In Fig. 3(B), the corresponding parameters for constant current (65 μA) at pH 1.5 for the same CD in the same concentrations are shown. These results can be regarded as representative also for the other uncharged CDs tested in the present work. Corresponding experiments at pH 7.4 gave similar results.

The best registrations at pH 7.4 concerning isoform separability for the horse kidney MT preparation, were found for 2-hydroxypropyl-α-CD (Fig. 4). At this pH Cd is bound to the protein. The identification of the MT forms appears by comparing the registrations at 200 and 254 nm. Homogenous Cd-MT displays optimal metal-thiolate absorption at 254 nm. As MT does not contain aromatic amino acids, the presence of absorption at 254 nm and the absence of absorption at 280 nm can be used as a convenient evidence for Cd containing MTs [2]. Fig. 4C shows that the MT-IB form consists of two subforms designated as IB1 and IB2, while the IA form consists of at least three subforms of which the most prominent are the IA1, IA2 and IA3 forms. It may, therefore, be concluded that the presence of 2-hydroxypropyl-α-CD in the metal-binding state in fact improves the separability of the MT isoforms and subforms. At this pH the b form, however, which is not separated in its two subforms b1 and b2 for these conditions, appears ahead of the a forms. This has also been shown previously [2].

Previous data [2] show that the horse kidney MT-IB form moves faster in the capillary than the MT-IA form. Figs. 2(A) and 4(A) in the present work show that this is also true during the presence of CD (Figs. 2(B,C) and 4(B,C).

The present work shows that this is also true during the presence of CD, which indicate that CD affects the two MT forms in a similar way. The main isoform patterns are not affected either. This may not be surprising, however, because of the close similarity between the two forms both with respect to amino acid composition and monomeric molecular weight [16]. It has also been shown previously [2,5] that at pH 1.5 the MT-IB forms are more positively charged than the MT-IA forms, while above the isoelectric point at pH 7.4, when the metals are bound to the protein, the MT-IB forms are more negatively charged than the IA forms [2,5,17]. These properties are not affected either by the presence of CD in the capillary. As already mentioned the hydrophobic parts of the MT forms are hidden within the CD buckets leaving the hydrophilic groups to the watery outside environment. This probably makes the complex between the protein and the CD molecule more freely soluble and mobile, thereby improving the resolution in the capillary of the various forms in the horse kidney MT sample, as indicated in Fig. 4. The protein inclusion into the CD is probably temporary and takes place several times during the run. The separation could therefore also be improved because the different forms may remain in the cavity for various times.

In Fig. 5, the normalized migration times and peak heights at pH 1.5 are plotted for the various molecular forms in the horse kidney MT preparation in the presence of different concentrations of heptakis (2,6-di-*o*-methyl)-β-CD, measured both at 10 kV constant voltage and at 65 μA constant current. In both registration modes, the b1 and b2 forms showed a relatively slower migration times with increasing CD concentrations compared to the other forms, but not so marked with constant current. These particular forms also showed a considerable increase in peak heights for both registration modes at increasing CD. It is concluded that this may be caused by a higher hydrophobicity of these forms, thereby accomodating better into the CD buckets. The hydrophobicity of the various molecular forms may therefore serve as a

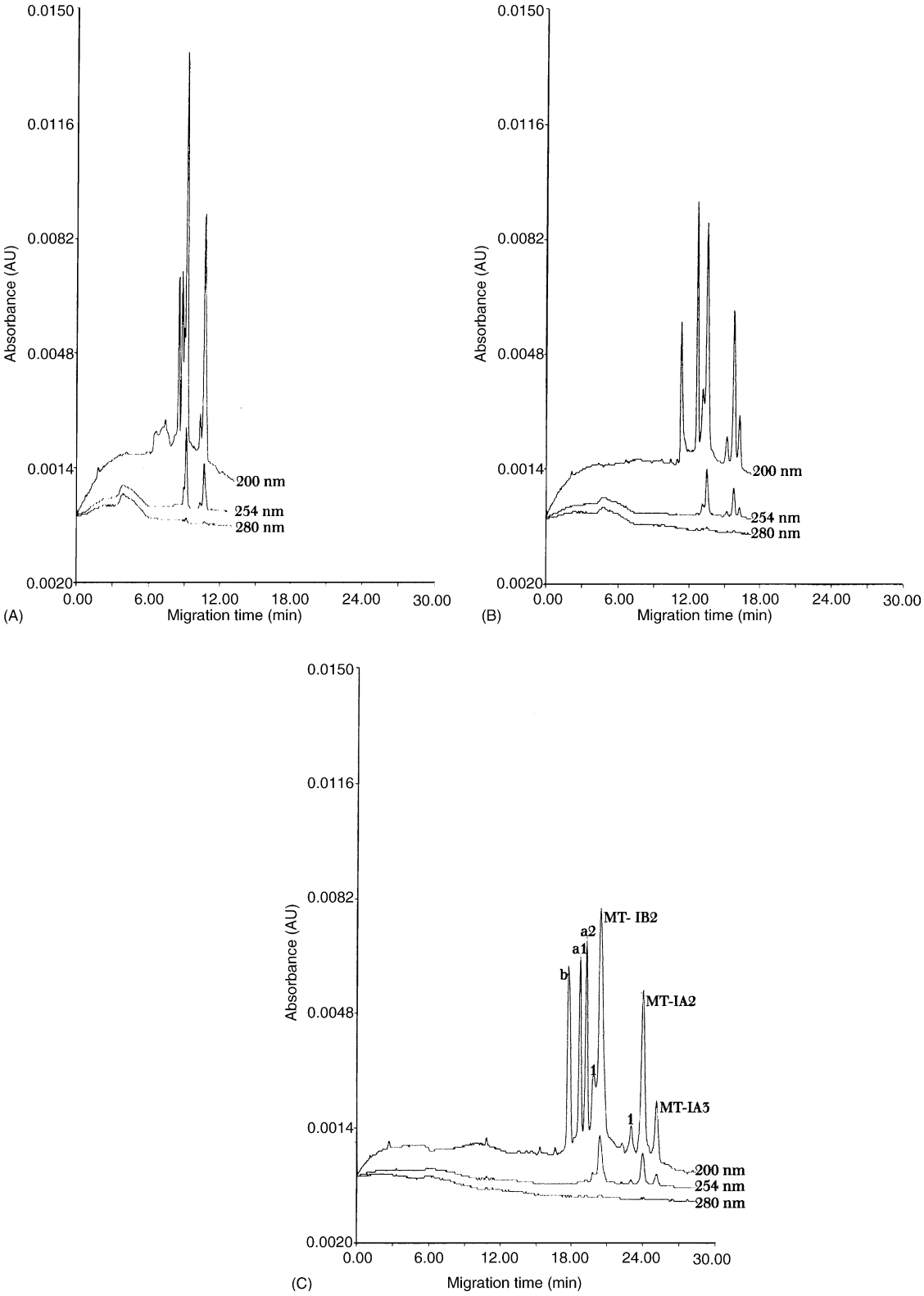

Fig. 4. CZE of the horse kidney MT preparation at 100 mM phosphate buffer, pH 7.4, 15 kV constant voltage. Polarity − to +. Otherwise as in Fig. 1. The identification of the various peaks are given in (C): (A) CD not added; (B) 8% 2-hydroxypropyl-α-CD; (C) 16%, otherwise as in (B).

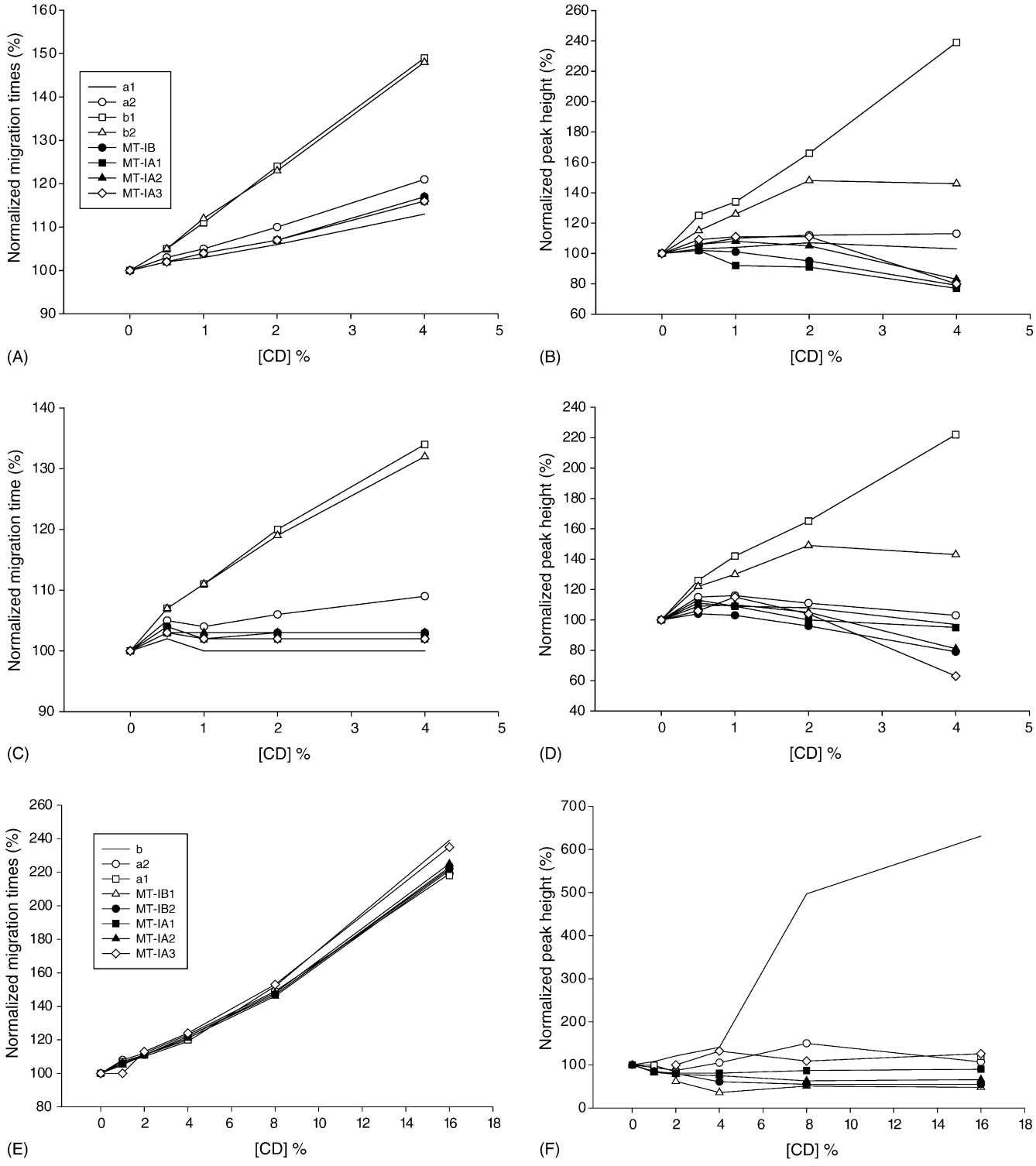

Fig. 5. Normalized migration times and peak heights of MT forms in the presence of various concentrations of CD: Heptakis (2,6-di-o-methyl)-β-CD at pH 1.5, 10 kV, migration times (A) and peak heights (B). Heptakis (2,6-di-*o*-methyl)-β-CD at pH 1.5, 65 μA, migration times (C) and peak heights (D). 2-Hydroxypropyl-α-CD at pH 7.4, 15 kV, migration times (E) and peak heights (F).

mean for their separation in these experiments. As shown in Fig. 4 there was also a slight reduction in the peak heights at the highest CD concentrations tested. This effect was generally seen for all the CDs tested in the present work. A possible explanation could be that at high CD concentrations, an electroendosmotic flow is generated toward the cathode, caused by complexation between the CD and the polyacrylamide coating of the capillary wall. If such an interaction

exists, it would be more pronounced at high CD concentrations.

For all the tested CDs reductions in migration times were found to be particularly pronounced for the b1 and b2 forms. Apart from the small b1 form, the peak heights were little affected by the presence of CD. The CDs tested seemed to have the ability to elute b1 from the b2 peak, thereby showing a large percentage increase in peak height compared to the situation without CD presence, not necessarily affecting the peak areas. For the γ-CD polymer and the hydroxypropyl-γ-CD, however, the strong initial increase observed at low CD concentrations, was followed by a gradual decrease. These data are not shown.

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